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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Jørgensen et al. Confirmation No: 9117

Serial No.: 09/928,847 Group Art Unit: 1636

Filed: August 13, 2001 Examiner: D. Lambertson

For: Method For Stable Chromosomal Multi-Copy Integration Of Genes

CERTIFICATE OF MAILING UNDER 37 CFR 1.8(a)

Commissioner for Patents
P.O. Box 1450
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TRANSMITTAL OF CERTIFIED COPY OF PRIORITY APPLICATION(S)

Commissioner for Patents
P.O. Box 1450
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Sir:

Attached please find a certified copy of the foreign application from which priority is claimed for this case:

Country: Denmark
Application Number: PA 2000 00981
Filing Date: 23 June 2000

Respectfully submitted,

Date: June 10, 2003

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Patent application No.: PA 2000 00981

Date of filing: 23 June 2000

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Novo Alle
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Denmark

Title: Method for stable chromosomal multi-copy integration of genes.

IPC: C 12 N 15/00

The attached documents are exact copies of the filed application

Patent- og Varemærkestyrelsen
Kongeriget Danmark

Patent- og Varemærkestyrelsen
Økonomi- og Erhvervsministeriet

20 May 2003

Helle Schackinger Olesen

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PATENT- G VAREMÆRKESTYRELSEN

Method for stable chromosomal multi-copy integration of gen s

Field of the Invention

The invention relates to a method for inserting genes into the chromosome of bacterial strains, and the resulting strains. In the biotech industry it is desirable to construct polypeptide production strains having several copies of a gene of interest stably chromosomally integrated, without leaving antibiotic resistance marker genes in the strains.

Background of the Invention

In the industrial production of polypeptides it is of interest to achieve a product yield as high as possible. One way to increase the yield is to increase the copy number of a gene encoding a polypeptide of interest. This can be done by placing the gene on a high copy number plasmid, however plasmids are unstable and are often lost from the host cells if there is no selective pressure during the cultivation of the host cells. Another way to increase the copy number of the gene of interest is to integrate it into the host cell chromosome in multiple copies. It has previously been described how to integrate a gene into the chromosome by double homologous recombination without using antibiotic markers (Hone *et al.*, Microbial Pathogenesis, 1988, 5: 407-418); integration of two genes has also been described (Novo Nordisk: WO 91/09129 and WO 94/14968). A problem with integrating several copies of a gene into the chromosome of a host cell is instability. Due to the sequence identity of the copies there is a high tendency for the them to recombine out of the chromosome again during cultivation of the host cell unless a selective marker or other essential DNA is included between the copies and selective pressure is applied during cultivation, especially if the genes are located in relative close vicinity of each other. It has been described how to integrate two genes closely spaced in anti-parallel tandem to achieve better stability (Novo Nordisk: WO 99/41358).

The present debate concerning the industrial use of recombinant DNA technology has raised some questions and concern about the use of antibiotic marker genes. Antibiotic marker genes are traditionally used as a means to select for strains carrying multiple copies of both the marker genes and an accompanying expression cassette coding for a polypeptide of industrial interest. In order to comply with the current demand for recombinant production host strains devoid of antibiotic markers, we have looked for possible alternatives to the present technology that will allow substitution of the antibiotic markers we use today with non-antibiotic marker genes. Thus in order to provide recombinant production strains devoid of antibiotic

resistance markers, it remains of industrial interest to find new methods to stably integrate genes in multiple copies into host cell chromosomes.

Summary of the Invention

The present invention solves the problem of integrating multiple copies of a gene of interest by homologous recombination into well defined chromosomal positions of a bacterial host strain which already comprises at least one copy of the gene of interest in a different position. This is done by making a deletion of part of a conditionally essential gene (hereafter called the "integration gene") in the host chromosome (or by integrating a partial gene into the host chromosome) of a strain which already comprises at least one copy of a gene of interest, so that the resulting strain has a deficiency, or a growth requirement, or is sensitive to a given stress. The next (i.e. second or third etc.) copy of the gene of interest is then introduced on a vector, on which the gene is flanked upstream by a partial fragment of the integration gene, and downstream is flanked by a fragment homologous to a DNA sequence downstream of the integration gene on the host chromosome. Thus, neither host chromosome nor the incoming vector contain a full version of the integration gene. In a non-limiting example the host chromosome may comprise the first two thirds of the integration gene and the vector the last two thirds, effectively establishing a sequence overlap of one third of the integration gene on the vector and the chromosome.

Expression of the full version of the integration gene will only occur if homologous recombination between vector and host chromosome takes place via the partial integration gene sequences, and this particular recombination event can be efficiently selected for, even against the background of homologous integration into the chromosome directed by the gene of interest into the identical gene(s) comprised on the chromosome already.

This strategy will enable directed gene integration by homologous recombination at predetermined loci, even though extended homology exists between the gene of interest on the incoming vector and other copies of this gene at other locations in the chromosome, and even though it is not feasible to identify the desired integrants based on the qualitative phenotype resulting from expression of the gene of interest, as this gene is already present in one or more copies in the host.

In a non-limiting example herein a *Bacillus* enzyme production strain is provided that comprise two anti-parallel copies (inverted orientation) of a gene encoding the commercially available amylase Termamyl® (Novo Nordisk, Denmark). A gene homologous to the *dal* gene of *Bacillus subtilis*, encoding a D-alanine racemase, was identified in the *Bacillus* production strain, it was

sequenced and a partial deletion was made in the *dal* gene of the *Bacillus* two-copy Termamyl® strain. A vector was constructed to effect a stable non-tandem chromosomal insertion of a third Termamyl® gene copy adjacent to the *dal locus*, in the process effectively restoring the complete *dal* gene, according to the above strategy.

In another non-limiting example herein, an additional copy of the amylase encoding gene was introduced into the xylose isomerase operon of the *Bacillus* enzyme production strain which already comprised at least two copies of the amylase gene located elsewhere on the chromosome.

Also in a non-limiting example we demonstrate the method of the invention by integrating an additional amylase-encoding gene into the gluconat operon of the *Bacillus* enzyme production strain.

Accordingly in a first aspect the invention relates to a method for constructing a cell comprising at least two copies of a gene of interest stably integrated into the chromosome in different positions, the method comprising the steps of:

a) providing a host cell comprising at least one chromosomal copy of the gene of interest;

b) altering a conditionally essential chromosomal gene(s) of the host cell whereby the gene becomes non-funtional;

c) making a DNA construct comprising:

i) an altered non-functional copy of the chromosomal gene(s) of step b); and

ii) at least one copy of the gene of interest flanked on one side by i) and on the other side by a DNA fragment homologous to a host cell DNA sequence adjacent to the gene(s) of step b); wherein a first recombination between the altered copy of i) and the altered chromomosomal gene(s) of step b) restores the chromosomal gene(s) to functionality and renders the cell selectable;

d) introducing the DNA construct into the host cell and cultivating the cell under conditions that require a functional gene(s) of step b); and

e) selecting a host cell that grows under the conditions of step d); wherein the at least one copy of the gene of interest has integrated into the host cell chromosome adjacent to the gene(s) of step b); and optionally

f) repeating steps a) to e) at least once using a different chromosomal gene(s) in step b) in each repeat.

Herein genetic tools are also described in the form of DNA constructs necessary for carrying out the method of the invention.

Consequently in a second aspect the invention relates to a DNA construct comprising:

i) an altered non-functional copy of a conditionally essential chromosomal gene(s) from a host cell, preferably the copy is partially deleted; and

ii) at least one copy of a gene of interest flanked on one side by i) and on the other side by a DNA fragment homologous to a host cell DNA sequence located on the host cell chromosome adjacent to the conditionally essential gene(s) of i).

The present invention provides a method for obtaining a host cell comprising at least two copies of a gene of interest stably integrated on the chromosome adjacent to conditionally essential *loci*.

Accordingly in a third aspect the invention relates to a host cell comprising at least two copies of a gene of interest stably integrated into the chromosome, where each copy is integrated adjacent to different conditionally essential *loci*, wherein the cell is obtainable by any of the methods defined in claims 1 - 18.

The method of the invention relies on complementing a conditionally essential gene(s) that was rendered non-functional, and a number of suitable host cells comprising such non-functional genes are described herein. To carry out multiple rounds of gene integration according to the invention it is advantageous to provide a host cell comprising several non-functional conditionally essential genes.

In a fourth aspect the invention relates to a *Bacillus licheniformis* cell, wherein at least two conditionally essential genes are rendered non-functional, preferably the genes are chosen from the group consisting of *xylR*, *xylA*, *galE*, *gntR*, *gntK*, *gntP*, *gntZ*, and *dal*.

Any host cell as described herein for use in a method of the invention is intended to be encompassed by the scope of the invention.

Consequently in a fifth aspect the invention relates to the use of a cell as defined in the previous aspect in a method as defined in the first aspect.

As mentioned above, genetic tools of the invention are described herein, and it is intended that the scope of the invention comprises such constructs when present in or propagated in host cells as is common in the art.

Yet another aspect of the invention relates to a cell comprising a DNA construct as defined in the second aspect.

In a final aspect the invention relates to a process for producing an enzyme of interest, comprising cultivating a cell as defined in any of the preceding aspects under conditions appropriate for producing the enzyme, and optionally purifying the enzyme.

Figures

Figure 1: Schematic representation of the *B. licheniformis* xylose isomerase region,
PCR fragments, Deletion and Integration plasmids and strains.

Figure 2: Schematic representation of the *B. licheniformis* gluconat region,
PCR fragments, Deletion and Integration plasmids and strains.

Figure 3: Schematic representation of the *B. licheniformis* D-alanine racemase encoding region, PCR fragments, Deletion and Integration plasmids and strains.

Definitions

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook et al., 1989") *DNA Cloning: A Practical Approach*, Volumes I and II /D.N. Glover ed. 1985); *Oligonucleotide Synthesis* (M.J. Gait ed. 1984); *Nucleic Acid Hybridization* (B.D. Hames & S.J. Higgins eds (1985)); *Transcription And Translation* (B.D. Hames & S.J. Higgins, eds. (1984)); *Animal Cell Culture* (R.I. Freshney, ed. (1986)); *Immobilized Cells And Enzymes* (IRL Press, (1986)); B. Perbal, *A Practical Guide To Molecular Cloning* (1984).

A "polynucleotide" is a single- or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. Polynucleotides include RNA and DNA, and may be isolated from natural sources, synthesized *in vitro*, or prepared from a combination of natural and synthetic molecules.

A "nucleic acid molecule" or "nucleotide sequence" refers to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine; "RNA molecules") or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; "DNA molecules") in either single stranded form, or a double-stranded helix. Double stranded DNA-DNA, DNA-RNA and RNA-RNA helices are possible. The term nucleic acid molecule, and in particular DNA or RNA molecule, refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary or quaternary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear or circular DNA molecules (e.g., restriction fragments), plasmids, and chromosomes. In discussing the

structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (i.e., the strand having a sequence homologous to the mRNA). A "recombinant DNA molecule" is a DNA molecule that has undergone a molecular biological manipulation.

A DNA "coding sequence" or an "open reading frame (ORF)" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide in a cell *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences. The ORF "encodes" the polypeptide. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. If the coding sequence is intended for expression in a eukaryotic cell, a polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

An expression vector is a DNA molecule, linear or circular, that comprises a segment encoding a polypeptide of interest operably linked to additional segments that provide for its transcription. Such additional segments may include promoter and terminator sequences, and optionally one or more origins of replication, one or more selectable markers, an enhancer, a polyadenylation signal, and the like. Expression vectors are generally derived from plasmid or viral DNA, or may contain elements of both.

Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, terminators, and the like, that provide for the expression of a coding sequence in a host cell. In eukaryotic cells, polyadenylation signals are control sequences.

A "secretory signal sequence" is a DNA sequence that encodes a polypeptide (a "secretory peptide" that, as a component of a larger polypeptide, directs the larger polypeptide through a secretory pathway of a cell in which it is synthesized. The larger polypeptide is commonly cleaved to remove the secretory peptide during transit through the secretory pathway.

The term "promoter" is used herein for its art-recognized meaning to denote a portion of a gene containing DNA sequences that provide for the binding of RNA polymerase and initiation of transcription. Promoter sequences are commonly, but not always, found in the 5' non-coding regions of genes.

A chromosomal gene is rendered "non-functional" if the polypeptide that the gene encodes can no longer be expressed in a functional form. Such non-functionality of a gene can be induced by a wide variety of genetic manipulations as known in the art, some of which are described in Sambrook *et al. vide supra*. Partial deletions within the ORF of a gene will often render the gene non-functional, as will mutations.

"Operably linked", when referring to DNA segments, indicates that the segments are arranged so that they function in concert for their intended purposes, e.g. transcription initiates in the promoter and proceeds through the coding segment to the terminator.

A coding sequence is "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then trans-RNA spliced and translated into the protein encoded by the coding sequence.

"Heterologous" DNA refers to DNA not naturally located in the cell, or in a chromosomal site of the cell. Preferably, the heterologous DNA includes a gene foreign to the cell.

As used herein the term "nucleic acid construct" is intended to indicate any nucleic acid molecule of cDNA, genomic DNA, synthetic DNA or RNA origin. The term "construct" is intended to indicate a nucleic acid segment which may be single- or double-stranded, and which may be based on a complete or partial naturally occurring nucleotide sequence encoding a polypeptide of interest. The construct may optionally contain other nucleic acid segments.

The nucleic acid construct of the invention encoding the polypeptide of the invention may suitably be of genomic or cDNA origin, for instance obtained by preparing a genomic or cDNA library and screening for DNA sequences coding for all or part of the polypeptide by hybridization using synthetic oligonucleotide probes in accordance with standard techniques (cf. Sambrook et al., supra).

The nucleic acid construct of the invention encoding the polypeptide may also be prepared synthetically by established standard methods, e.g. the phosphoamidite method described by Beaucage and Caruthers, Tetrahedron Letters 22 (1981), 1859 - 1869, or the method described by Matthes et al., EMBO Journal 3 (1984), 801 - 805. According to the phosphoamidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in suitable vectors.

Furthermore, the nucleic acid construct may be of mixed synthetic and genomic, mixed synthetic and cDNA or mixed genomic and cDNA origin prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate), the fragments corresponding to various parts of the entire nucleic acid

construct, in accordance with standard techniques. The nucleic acid construct may also be prepared by polymerase chain reaction using specific primers, for instance as described in US 4,683,202 or Saiki et al., Science 239 (1988), 487 - 491.

The term nucleic acid construct may be synonymous with the term "expression cassette" when the nucleic acid construct contains the control sequences necessary for expression of a coding sequence of the present invention

The term "control sequences" is defined herein to include all components that are necessary or advantageous for expression of the coding sequence of the nucleic acid sequence. Each control sequence may be native or foreign to the nucleic acid sequence encoding the polypeptide. Such control sequences include, but are not limited to, a leader, a polyadenylation sequence, a propeptide sequence, a promoter, a signal sequence, and a transcription terminator. At a minimum, the control sequences include a promoter, and transcriptional and translational stop signals. The control sequences may be provided with linkers for the purpose of introducing specific restriction sites facilitating ligation of the control sequences with the coding region of the nucleic acid sequence encoding a polypeptide.

The control sequence may be an appropriate promoter sequence, a nucleic acid sequence that is recognized by a host cell for expression of the nucleic acid sequence. The promoter sequence contains transcription and translation control sequences that mediate the expression of the polypeptide. The promoter may be any nucleic acid sequence that shows transcriptional activity in the host cell of choice and may be obtained from genes encoding extracellular or intracellular polypeptides either homologous or heterologous to the host cell.

The control sequence may also be a suitable transcription terminator sequence, a sequence recognized by a host cell to terminate transcription. The terminator sequence is operably linked to the 3' terminus of the nucleic acid sequence encoding the polypeptide. Any terminator which is functional in the host cell of choice may be used in the present invention.

The control sequence may also be a polyadenylation sequence, a sequence which is operably linked to the 3' terminus of the nucleic acid sequence and which, when transcribed, is recognized by the host cell as a signal to add polyadenosine residues to transcribed mRNA. Any polyadenylation sequence which is functional in the host cell of choice may be used in the present invention.

The control sequence may also be a signal peptide-coding region, which codes for an amino acid sequence linked to the amino terminus of the

polypeptide which can direct the expressed polypeptide into the cell's secretory pathway of the host cell. The 5' end of the coding sequence of the nucleic acid sequence may inherently contain a signal peptide-coding region naturally linked in translation reading frame with the segment of the coding region which encodes the secreted polypeptide. Alternatively, the 5' end of the coding sequence may contain a signal peptide-coding region which is foreign to that portion of the coding sequence which encodes the secreted polypeptide. A foreign signal peptide-coding region may be required where the coding sequence does not normally contain a signal peptide-coding region. Alternatively, the foreign signal peptide coding region may simply replace the natural signal peptide coding region in order to obtain enhanced secretion of the polypeptide relative to the natural signal peptide coding region normally associated with the coding sequence. The signal peptide-coding region may be obtained from a glucoamylase or an amylase gene from an *Aspergillus* species, a lipase or proteinase gene from a *Rhizomucor* species, the gene for the alpha-factor from *Saccharomyces cerevisiae*, an amylase or a protease gene from a *Bacillus* species, or the calf preprochymosin gene. However, any signal peptide coding region capable of directing the expressed polypeptide into the secretory pathway of a host cell of choice may be used in the present invention.

The control sequence may also be a propeptide coding region, which codes for an amino acid sequence positioned at the amino terminus of a polypeptide. The resultant polypeptide is known as a proenzyme or propolypeptide (or a zymogen in some cases). A propolypeptide is generally inactive and can be converted to mature active polypeptide by catalytic or autocatalytic cleavage of the propeptide from the propolypeptide. The propeptide coding region may be obtained from the *Bacillus subtilis* alkaline protease gene (*aprE*), the *Bacillus subtilis* neutral protease gene (*nprT*), the *Saccharomyces cerevisiae* alpha-factor gene, or the *Myceliophthora thermophilum* laccase gene (WO 95/33836).

It may also be desirable to add regulatory sequences which allow the regulation of the expression of the polypeptide relative to the growth of the host cell. Examples of regulatory systems are those which cause the expression of the gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Regulatory systems in prokaryotic systems would include the *lac*, *tac*, and *trp* operator systems. Other examples of regulatory sequences are those which allow for gene amplification. In eukaryotic systems, these include the dihydrofolate reductase gene which is amplified in the presence of methotrexate, and the metallothionein genes which are amplified with heavy

metals. In these cases, the nucleic acid sequence encoding the polypeptide would be placed in tandem with the regulatory sequence.

Examples of suitable promoters for directing the transcription of the nucleic acid constructs of the present invention, especially in a bacterial host cell, are the promoters obtained from the *E. coli lac* operon, the *Streptomyces coelicolor* agarase gene (*dagA*), the *Bacillus subtilis* levansucrase gene (*sacB*), the *Bacillus subtilis* alkaline protease gene, the *Bacillus licheniformis* alpha-amylase gene (*amyL*), the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), the *Bacillus amyloliquefaciens* alpha-amylase gene (*amyQ*), the *Bacillus amyloliquefaciens* BAN AMYLASE GENE, the *Bacillus licheniformis* penicillinase gene (*penP*), the *Bacillus subtilis xylA* and *xylB* genes, and the prokaryotic beta-lactamase gene (Villa-Kamaroff *et al.*, 1978, *Proceedings of the National Academy of Sciences USA* 75:3727-3731), as well as the *tac* promoter (DeBoer *et al.*, 1983, *Proceedings of the National Academy of Sciences USA* 80:21-25). Further promoters are described in "Useful proteins from recombinant bacteria" in *Scientific American*, 1980, 242:74-94; and in Sambrook *et al.*, 1989, *supra*.

The present invention also relates to recombinant expression vectors comprising a nucleic acid sequence of the present invention, a promoter, and transcriptional and translational stop signals. The various nucleic acid and control sequences described above may be joined together to produce a recombinant expression vector which may include one or more convenient restriction sites to allow for insertion or substitution of the nucleic acid sequence encoding the polypeptide at such sites. Alternatively, the nucleic acid sequence of the present invention may be expressed by inserting the nucleic acid sequence or a nucleic acid construct comprising the sequence into an appropriate vector for expression. In creating the expression vector, the coding sequence is located in the vector so that the coding sequence is operably linked with the appropriate control sequences for expression, and possibly secretion.

The recombinant expression vector may be any vector (*e.g.*, a plasmid or virus) which can be conveniently subjected to recombinant DNA procedures and can bring about the expression of the nucleic acid sequence. The choice of the vector will typically depend on the compatibility of the vector with the host cell into which the vector is to be introduced. The vectors may be linear or closed circular plasmids. The vector may be an autonomously replicating vector, *i.e.*, a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, *e.g.*, a plasmid, an extrachromosomal element, a minichromosome, or an artificial chromosome. The vector may contain any means for assuring self-replication. Alternatively, the vector may be one which, when introduced

into the host cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. The vector system may be a single vector or plasmid or two or more vectors or plasmids which together contain the total DNA to be introduced into the genome of the host cell, or a transposon.

The vectors of the present invention preferably contain one or more "selectable markers" which permit easy selection of transformed cells. A selectable marker is a gene the product of which provides for biocide, antibiotic or viral resistance, resistance to heavy metals, prototrophy to auxotrophs, and the like.

A "conditionally essential gene" may function as a "non-antibiotic selectable marker". Non-limiting examples of bacterial conditionally essential selectable markers are the *dal* genes from *Bacillus subtilis* or *Bacillus licheniformis*, that are only essential when the bacterium is cultivated in the absence of D-alanine. Also the genes encoding enzymes involved in the turnover of UDP-galactose can function as conditionally essential markers in a cell when the cell is grown in the presence of galactose or grown in a medium which gives rise to the presence of galactose. Non-limiting examples of such genes are those from *B. subtilis or B. licheniformis* encoding UTP-dependent phosphorylase (EC 2.7.7.10), UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), or UDP-galactose epimerase (EC 5.1.3.2). Also the xylose isomerase genes, *xylR* and *xylA*, of *Bacilli* can be used as selectable markers in cells grown in minimal medium with xylose as sole carbon source. The genes necessary for utilizing gluconate, *gntR*, *gntK*, *gntP*, and *gntZ* can also be used as selectable markers in cells grown in minimal medium with gluconate as sole carbon source

Antibiotic selectable markers confer antibiotic resistance to such antibiotics as ampicillin, kanamycin, chloramphenicol, tetracycline, neomycin, hygromycin or methotrexate.

Furthermore, selection may be accomplished by co-transformation, *e.g.*, as described in WO 91/17243, where the selectable marker is on a separate vector.

The vectors of the present invention preferably contain an element(s) that permits stable integration of the vector, or of a smaller part of the vector, into the host cell genome or autonomous replication of the vector in the cell independent of the genome of the cell.

The vectors, or smaller parts of the vectors such as amplification units of the present invention, may be integrated into the host cell genome when introduced into a host cell. For chromosomal integration, the vector may rely on the nucleic acid sequence encoding the polypeptide or any other

element of the vector for stable integration of the vector into the genome by homologous or nonhomologous recombination.

Alternatively, the vector may contain additional nucleic acid sequences for directing integration by homologous recombination into the genome of the host cell. The additional nucleic acid sequences enable the vector to be integrated into the host cell genome at a precise location(s) in the chromosome(s). To increase the likelihood of integration at a precise location, the integrational elements should preferably contain a sufficient number of nucleic acids, such as 100 to 1,500 base pairs, preferably 400 to 1,500 base pairs, and most preferably 800 to 1,500 base pairs, which are highly homologous with the corresponding target sequence to enhance the probability of homologous recombination. The integrational elements may be any sequence that is homologous with the target sequence in the genome of the host cell. Furthermore, the integrational elements may be non-encoding or encoding nucleic acid sequences.

On the other hand, the vector may be integrated into the genome of the host cell by non-homologous recombination. These nucleic acid sequences may be any sequence that is homologous with a target sequence in the genome of the host cell, and, furthermore, may be non-encoding or encoding sequences. The copy number of a vector, an expression cassette, an amplification unit, a gene or indeed any defined nucleotide sequence is the number of identical copies that are present in a host cell at any time. A gene or another defined chromosomal nucleotide sequence may be present in one, two, or more copies on the chromosome. An autonomously replicating vector may be present in one, or several hundred copies per host cell.

For autonomous replication, the vector may further comprise an origin of replication enabling the vector to replicate autonomously in the host cell in question. Examples of bacterial origins of replication are the origins of replication of plasmids pBR322, pUC19, pACYC177, pACYC184, pUB110, pE194, pTA1060, and pAMß1. The origin of replication may be one having a mutation which makes its functioning temperature-sensitive in the host cell (see, *e.g.*, Ehrlich, 1978, *Proceedings of the National Academy of Sciences USA* 75:1433).

The present invention also relates to recombinant host cells, comprising a nucleic acid sequence of the invention, which are advantageously used in the recombinant production of the polypeptides. The term "host cell" encompasses any progeny of a parent cell which is not identical to the parent cell due to mutations that occur during replication.

The cell is preferably transformed with a vector comprising a nucleic acid sequence of the invention followed by integration of the vector into the host chromosome. "Transformation" means introducing a vector comprising

a nucleic acid sequence of the present invention into a host cell so that the vector is maintained as a chromosomal integrant or as a self-replicating extra-chromosomal vector. Integration is generally considered to be an advantage as the nucleic acid sequence is more likely to be stably maintained in the cell. Integration of the vector into the host chromosome may occur by homologous or non-homologous recombination as described above.

The choice of a host cell will to a large extent depend upon the gene encoding the polypeptide and its source. The host cell may be a unicellular microorganism, *e.g.*, a prokaryote, or a non-unicellular microorganism, e.g., a eukaryote. Useful unicellular cells are bacterial cells such as gram positive bacteria including, but not limited to, a *Bacillus* cell, *e.g.*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus coagulans*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus stearothermophilus*, *Bacillus subtilis*, and *Bacillus thuringiensis*; or a *Streptomyces* cell, *e.g.*, *Streptomyces lividans* or *Streptomyces murinus*, or gram negative bacteria such as *E. coli* and *Pseudomonas* sp. In a preferred embodiment, the bacterial host cell is a *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus stearothermophilus* or *Bacillus subtilis* cell.

The transformation of a bacterial host cell may, for instance, be effected by protoplast transformation (see, *e.g.*, Chang and Cohen, 1979, *Molecular General Genetics* 168:111-115), by using competent cells (see, *e.g.*, Young and Spizizin, 1961, *Journal of Bacteriology* 81:823-829, or Dubnar and Davidoff-Abelson, 1971, *Journal of Molecular Biology* 56:209-221), by electroporation (see, *e.g.*, Shigekawa and Dower, 1988, *Biotechniques* 6:742-751), or by conjugation (see, *e.g.*, Koehler and Thorne, 1987, *Journal of Bacteriology* 169:5771-5278).

The transformed or transfected host cells described above are cultured in a suitable nutrient medium under conditions permitting the expression of the desired polypeptide, after which the resulting polypeptide is recovered from the cells, or the culture broth.

The medium used to culture the cells may be any conventional medium suitable for growing the host cells, such as minimal or complex media containing appropriate supplements. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. in catalogues of the American Type Culture Collection). The media are prepared using procedures known in the art (see, *e.g.*, references for bacteria and yeast; Bennett, J.W. and LaSure, L., editors, *More Gene Manipulations in Fungi*, Academic Press, CA, 1991).

If the polypeptide is secreted into the nutrient medium, the polypeptide can be recovered directly from the medium. If the polypeptide

is not secreted, it is recovered from cell lysates. The polypeptide are recovered from the culture medium by conventional procedures including separating the host cells from the medium by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulphate, purification by a variety of chromatographic procedures, e.g. ion exchange chromatography, gelfiltration chromatography, affinity chromatography, or the like, dependent on the type of polypeptide in question.

The polypeptides may be detected using methods known in the art that are specific for the polypeptides. These detection methods may include use of specific antibodies, formation of an enzyme product, or disappearance of an enzyme substrate. For example, an enzyme assay may be used to determine the activity of the polypeptide.

The polypeptides of the present invention may be purified by a variety of procedures known in the art including, but not limited to, chromatography (*e.g.*, ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (*e.g.*, preparative isoelectric focusing (IEF), differential solubility (*e.g.*, ammonium sulfate precipitation), or extraction (see, *e.g.*, *Protein Purification*, J.-C. Janson and Lars Ryden, editors, VCH Publishers, New York, 1989).

Detailed description of the invention

A method for constructing a cell comprising at least two copies of a gene of interest stably integrated into the chromosome in different positions according to the first aspect of the invention.

In the method of the invention it is envisioned that after the directed and selectable integration of the DNA construct into the chromosome of the host cell by the first homologous recombination, a second recombination can take place between a DNA fragment comprised in the construct and a homologous host cell DNA sequence located adjecent to the gene(s) of step b) of the method of the first aspect, where the DNA fragment of the construct is homologous to said host cell DNA sequence.

Accordingly a preferred embodiment of the invention relates to the method of the first aspect, wherein subsequent to step d) and prior to step e) a second recombination takes place between the DNA fragment and the homologous host cell DNA sequence.

Further it is envisioned that one might add a marker gene to the DNA construct, which could ease selection of first recombination integrants, where the marker gene would be excised from the host cell chromosome again by the second recombination as described above.

In a preferred embodiment the invention relates to the method of the first aspect, where the DNA construct further comprises a marker gene which is located in the construct such that it is recombined out of the chromosome by the second recombination; preferably the marker gene confers resistance to an antibiotic, more preferably the antibiotic is chosen from the group consisting of chloramphenicol, kanamycin, ampillin, erythromycin, spectinomycin and tetracycline; and most preferably a host cell is selected which grows under the conditions of step d) of the first aspect and which does not contain the marker gene in the chromosome.

The method of the invention can also be carried out by including a marker gene in that part of the DNA construct which remains integrated in the chromosome after the second recombination event. However as it is preferred not to have marker genes in the chromosome, an alternative way of removing the marker gene must be employed after the integration has been carried out. Specific restriction enzymes or resolvases that excise portions of DNA, if it is flanked on both sides by certain recognition sequences known as resolvase sites or *res*-sites, are well known in the art.

A preferred embodiment of the invention relates to the method of the first aspect, where the DNA construct further comprises a marker gene located between the altered copy and the DNA fragment, and wherein the marker gene is flanked by nucleotide sequences that are recognized by a specific resolvase, preferably the nucleotide sequences are *res*; even more preferably the marker gene is excised from the chromosome by the action of a resolvase enzyme after step e) and prior to step f) in the first aspect.

The gene of interest may encode an enzyme that is naturally produced by the host cell, indeed one may simply want to increase the number of copies of a gene endogenous to the host cell.

Accordingly a preferred embodiment of the invention relates to the method of the first aspect, wherein the gene of interest originates from the host cell.

In another preferred embodiment the invention relates to the method of the first aspect, wherein the gene of interest encodes an enzyme, preferably an amylolytic enzyme, a lipolytic enzyme, a proteolytic enzyme, a cellulytic enzyme, an oxidoreductase or a plant cell-wall degrading enzyme, and more preferably an enzyme with an activity selected from the group consisting of aminopeptidase, amylase, amyloglucosidase, carbohydrase, carboxypeptidase, catalase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, esterase, galactosidase, beta-galactosidase, glucoamylase, glucose oxidase, glucosidase, haloperoxidase, hemicellulase, invertase, isomerase, laccase, ligase, lipase, lyase,

mannosidase, oxidase, pectinase, peroxidase, phytase, phenoloxidase, polyphenoloxidase, protease, ribonuclease, transferase, transglutaminase, or xylanase.

As mentioned above, the gene of interest may be endogenous to the host cell, however it may even be more advantageous if the production cell obtained by the method of the invention contains no foreign DNA at all, when the integration procedure is completed.

Yet a preferred embodiment of the invention relates to the method of the first aspect, wherein the host cell selected in step e) of the first aspect comprises DNA only of endogenous origin.

Many ways exist in the art of rendering a gene non-functional by manipulation, such as partially deleting the gene or the promoter of the gene, or by introducing mutations in the gene or the promoter region of the gene.

A preferred embodiment of the invention relates to the method of the first aspect, wherein the chromosomal gene(s) of the host cell is altered by partially deleting the gene, or by introducing mutations in the gene(s).

The present invention relies on rendering a chromosomal gene(s) of the host cell non-functional in a step, and in particular relies on a number of conditionally essential genes to be rendered non-functional. The gene(s) may be rendered non-functional by a partial deletion or a mutation as known in the art; specifically the gene(s) may be rendered non-functional through the use of a "Deletion plasmid(s)" as shown herein in non-limiting examples below. For each of the preferred embodiments relating to the altered chromosomal gene(s) of step b) of the method of the first aspect, the most preferred embodiment is shown by non-limiting examples herein and reference is made to the genetic tools constructed for that purpose, such as the PCR primer sequences used for constructing the "Deletion plasmid(s)".

Accordingly a preferred embodiment of the invention relates to the method of the first aspect, wherein the chromosomal gene(s) of the host cell that is altered in step b) encodes a D-alanine racemase, preferably the gene(s) is a *dal* homologue from a *Bacillus* cell, more preferably the gene is homologous to *dal* from *Bacillus subtilis*, and most preferably the gene(s) is the *dal* gene of *Bacillus licheniformis*.

Another preferred embodiment of the invention relates to the method of the first aspect, wherein the chromosomal gene(s) of the host cell that is altered in step b) encodes a D-alanine racemase and is at least 75% identical, preferably 80% identical, or preferably 85% identical, more preferably 90% identical, or more preferably 95% and most preferably at least 97% identical to the *dal* sequence of *Bacillus licheniformis* shown in positions 1303 to 2469 in SEQ ID 12.

A preferred embodiment of the invention relates to the method of the first aspect, wherein the chromosomal gene(s) of the host cell that is altered in step b) is one or more genes of a xylose isomerase operon, preferably the gene(s) is homologous to the *xylR* gene or the *xylA* gene from *Bacillus subtilis*, more preferably the gene(s) is homologous to both *xylR* and *xylA*, and most preferably the gene(s) is homologous to one or more genes of the xylose isomerase operon of *Bacillus licheniformis*.

A preferred embodiment of the invention relates to the method of the first aspect, wherein the chromosomal gene(s) of the host cell that is altered in step b) encodes a galactokinase (EC 2.7.1.6), an UTP-dependent pyrophosphorylase (EC 2.7.7.10), an UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), or an UDP-galactose epimerase (EC 5.1.2.3), preferably the gene(s) encodes an UDP-galactose epimerase (EC 5.1.2.3), more preferably the gene(s) is homologous to *galE* of a *Bacillus*, and most preferably the gene is *galE* of *Bacillus licheniformis*.

A preferred embodiment of the invention relates to the method of the first aspect, wherein the chromosomal gene(s) of the host cell that is altered in step b) is one or more genes of a gluconate operon, preferably the gene(s) encodes a gluconate kinase (EC 2.7.1.12) or a gluconate permease or both, more preferably the gene(s) is one or more genes homologous to genes from *Bacillus subtilis* chosen from the group consisting of *gntR*, *gntK*, *gntP*, and *gntZ*, and most preferably the gene(s) is one or more genes of *gntR*, *gntK*, *gntP*, and *gntZ* from *Bacillus licheniformis*.

As described herein the method of the invention is very relevant for the biotech industry and a number of preferred organisms are very well known in this industry, especially Gram positive host cells, and certainly host cells of the *Bacillus* genus, specifically *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus clausii*, *Bacillus coagulans*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus stearothermophilus*, *Bacillus subtilis*, and *Bacillus thuringiensis*.

A preferred embodiment of the invention relates to the method of the first aspect, wherein the host cell is a Gram-positive bacterial cell, preferably a *Bacillus* cell, and most preferably a *Bacillus* cell chosen from the group consisting of *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus clausii*, *Bacillus coagulans*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus stearothermophilus*, *Bacillus subtilis*, and *Bacillus thuringiensis*.

Another preferred embodiment of the invention relates to the method of the first aspect, wherein the DNA construct is a plasmid.

As described elsewhere herein, the present invention provides genetic tools for carrying out the method of the invention, such as host cells, and DNA constructs of the invention, such as a DNA construct of the second aspect comprising:

i) an altered non-functional copy of a conditionally essential chromosomal gene(s) from a host cell, preferably the copy is partially deleted; and

ii) at least one copy of a gene of interest flanked on one side by i) and on the other side by a DNA fragment homologous to a host cell DNA sequence located on the host cell chromosome adjecent to the conditionally essential gene(s) of i).

A preferred embodiment of the invention relates to the DNA construct of the second aspect, wherein the chromosomal gene(s) of the host cell that is altered in i) encodes a D-alanine racemase, preferably the gene(s) is a *dal* homologue from a *Bacillus* cell, more preferably the gene is homologous to *dal* from *Bacillus subtilis*, and most preferably the gene is the *dal* gene of *Bacillus licheniformis*.

Another preferred embodiment of the invention relates to the DNA construct of the second aspect, wherein the chromosomal gene(s) of the host cell that is altered in i) encodes a D-alanine racemase and is at least 75% identical, preferably 80% identical, or preferably 85% identical, more preferably 90% identical, or more preferably 95% and most preferably at least 97% identical to the *dal* sequence of *Bacillus licheniformis* shown in positions 1303 to 2469 in SEQ ID 12.

Yet another preferred embodiment of the invention relates to the DNA construct of the second aspect, wherein the chromosomal gene(s) of the host cell that is altered in i) is one or more genes of a xylose isomerase operon, preferably the gene(s) is homologous to the *xylR* gene or the *xylA* gene from *Bacillus subtilis*, more preferably the gene(s) is both a *xylR* and a *xylA* homologue, and most preferably the gene(s) is homologous to one or more genes of the xylose isomerase operon of *Bacillus licheniformis*.

Still another preferred embodiment of the invention relates to the DNA construct of the second aspect, wherein the chromosomal gene(s) of the host cell that is altered in i) encodes a galactokinase (EC 2.7.1.6), an UTP-dependent pyrophosphorylase (EC 2.7.7.10), an UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), or an UDP-galactose epimerase (EC 5.1.2.3), preferably the gene(s) encodes an UDP-galactose epimerase (EC 5.1.2.3), more preferably the gene(s) is homologous to the *galE* gene of *Bacillus subtilis*, and most preferably the gene(s) is the *galE* gene of *Bacillus licheniformis*.

One more preferred embodiment of the invention relates to the DNA construct of the second aspect, wherein the chromosomal gene(s) of the host cell that is altered in i) is one or more genes of a gluconate operon, preferably the gene(s) encodes a gluconate kinase (EC 2.7.1.12) or a gluconate permease or both, more preferably the gene(s) is homologous to one or more genes from *Bacillus subtilis* chosen from the group consisting of *gntR*, *gntK*, *gntP*, and *gntZ*, and most preferably the gene(s) is one or more genes of *gntR*, *gntK*, *gntP*, and *gntZ* from *Bacillus licheniformis*.

The present invention provides a method for constructing a production host cell that is very useful to the biotech industry, such as a host cell of the third aspect comprising at least two copies of a gene of interest stably integrated into the chromosome, where each copy is integrated adjecent to different conditionally essential *loci*, wherein the cell is obtainable by any of the methods defined in the first aspect.

The method of the first aspect describes the integration of a gene of interest into the chromosome of a host cell, so that the gene of interest is integrated in a position that is adjecent to the conditionally essential *locus*. The exact relative positions of the gene of interest and the *locus* are not of major relevance for the method, however generally speaking it is of interest to minimize the distance in basepairs separating the two, both to achieve a more stable integration, but also to minimize the integration of superfluous DNA sequence into the host cell genome.

Accordingly a preferred embodiment of the invention relates to the host cell of the third aspect, wherein the gene of interest is separated from the conditionally essential *locus* by no more than 1000 basepairs, preferably no more than 750 basepairs, more preferably no more than 500 basepairs, even more preferably no more than 250 basepairs, and most preferably no more than 100 basepairs.

As mentioned above, it is of interest to minimize the presence of integrated or superfluous DNA sequence in the host cell genome, especially DNA of exogenous origin, and the ideal host cell contains only DNA of endogenous origin such as multiple copies of an endogenous gene of interest integrated in different well defined chromosomal locations.

Consequently a preferred embodiment of the invention relates to the host cell of the third aspect, which contains only endogenous DNA.

Certain bacterial strains are preferred as host cells in the biotech industry as mentioned previously.

A preferred embodiment of the invention relates to the host cell of the third aspect, which is a Gram-positive bacterial cell, preferably a *Bacillus* cell, and most preferably a *Bacillus* cell chosen from the group consisting of *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus*

brevis, *Bacillus circulans*, *Bacillus clausii*, *Bacillus coagulans*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus stearothermophilus*, *Bacillus subtilis*, and *Bacillus thuringiensis*.

Another preferred embodiment of the invention relates to the host cell of the third aspect, wherein a copy of the gene of interest is integrated adjecent to a gene encoding a D-alanine racemase, preferably a gene homologous to the *dal* gene from *Bacillus subtilis*, more preferably a gene at least 75% identical to the *dal* sequence of *Bacillus licheniformis* shown in positions 1303 to 2469 in SEQ ID 12, even more preferably 80% identical, or even more preferably a gene at least 85% identical, still more preferably 90% identical, more preferably at least 95% identical, and most preferably at least 97% identical to the *dal* sequence of *Bacillus licheniformis* shown in positions 1303 to 2469 in SEQ ID 12.

Yet another preferred embodiment of the invention relates to the host cell of the third aspect, wherein a copy of the gene of interest is integrated adjecent to a gene of a xylose isomerase operon, preferably adjecent to genes homologous to the *xylR* or *xylA* genes from *Bacillus subtilis*, and most preferably adjecent to *xylR* or *xylA* from *Bacillus licheniformis*.

One more preferred embodiment of the invention relates to the host cell of the third aspect, wherein a copy of the gene of interest is integrated adjecent to a gene encoding a galactokinase (EC 2.7.1.6), an UTP-dependent pyrophosphorylase (EC 2.7.7.10), an UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), or an UDP-galactose epimerase (EC 5.1.2.3), preferably adjecent to a gene encoding an UDP-galactose epimerase (EC 5.1.2.3), more preferably adjecent to a gene homologous to the *galE* gene from *Bacillus subtilis*, and most preferably adjecent to *galE* from *Bacillus licheniformis*.

An additional preferred embodiment of the invention relates to the host cell of the third aspect, wherein a copy of the gene of interest is integrated adjecent to a gene of a gluconate operon, preferably adjecent to a gene that encodes a gluconate kinase (EC 2.7.1.12) or a gluconate permease, more preferably adjecent to a gene homologous to a *Bacillus subtilis* gene chosen from the group consisting of *gntR*, *gntK*, *gntP*, and *gntZ*, and most preferably adjecent to *gntR*, *gntK*, *gntP*, or *gntZ* from *Bacillus licheniformis*.

The host cell of the third aspect is especially interesting for the industrial production of polypeptides such as enzymes.

A preferred embodiment of the invention relates to the host cell of the third aspect, wherein the gene of interest encodes an enzyme, preferably an amylolytic enzyme, a lipolytic enzyme, a proteolytic enzyme, a cellulytic

enzyme, an oxidoreductase or a plant cell-wall degrading enzyme, and more preferably an enzyme selected from the group consisting of aminopeptidase, amylase, amyloglucosidase, carbohydrase, carboxypeptidase, catalase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, esterase, galactosidase, beta-galactosidase, glucoamylase, glucose oxidase, glucosidase, haloperoxidase, hemicellulase, invertase, isomerase, laccase, ligase, lipase, lyase, mannosidase, oxidase, pectinase, peroxidase, phytase, phenoloxidase, polyphenoloxidase, protease, ribonuclease, transferase, transglutaminase, or xylanase.

A further preferred embodiment of the invention relates to the host cell of the third aspect, wherein no antibiotic markers are present.

The present invention teaches the construction of host cells that are suitable for use in the method of the first aspect, especially host cells wherein one, two or more conditionally essential genes are rendered non-functional. In non-limiting examples below is shown how the preferred conditionally essential genes of the invention are rendered non-functional through a partial deletion by using specific Deletion Plasmids of the invention. Specifically the present invention relates to a *Bacillus licheniformis* cell of the fourth aspect, wherein at least two conditionally essential genes are rendered non-functional, preferably the genes are chosen from the group consisting of *xylR, xylA, galE, gntR, gntK, gntP, gntZ,* and *dal*.

The use of such a host cell of the fourth aspect is likewise envisioned in the method of the first aspect.

Another genetic tool provided by the present invention for the method of the first aspect, is a host cell comprising a DNA construct of the second aspect.

A final aspect of the invention relatest to a process for producing an enzyme of interest, comprising cultivating a cell of the third aspect under conditions appropriate for producing the enzyme, and optionally purifying the enzyme.

Examples

Example 1

Bacillus licheniformis SJ4671 (WO 99/41358) comprises two stably integrated *amyL* gene copies in its chromosome, inserted in opposite relative orientations in the region of the *B. licheniformis* alpha-amylase gene, *amyL*. The following example describes the insertion into this strain of a third *amyL* gene copy by selectable, directed integration into another defined

region of the *B. licheniformis* chromosome resulting in a strain comprising three stable chromosomal copies of the *amyL* gene but which is devoid of foreign DNA.

Xylose isomerase deletion/integration outline (Figure 1)

The sequence of the *Bacillus lichenformis* xylose isomerase region is available in GenBank/EMBL with accession number Z80222.

A plasmid denoted "Deletion plasmid" was constructed by cloning two PCR amplified fragments from the xylose isomerase region on a temperature-sensitive parent plasmid. The PCR fragments were denoted "A" and "B", wherein A comprises the *xylR* promoter and part of the *xylR* gene; and B comprises an internal fragment of *xylA* missing the promoter and the first 70 basepairs of the gene. A spectinomycin resistance gene flanked by resolvase (*res*) sites was introduced between fragments A and B on the plasmid. This spectinomycin resistance gene could later be removed by resolvase-mediated site-specific recombination.

The xylose isomerase deletion was transferred from the Deletion plasmid to the chromosome of a *Bacillus* target strain by double homologous recombination via fragments A and B, mediated by integration and excision of the temperature-sensitive plasmid. The resulting strain was denoted "Deletion strain". This strain is unable to grow on minimal media with xylose as sole carbon source.

An "Integration plasmid" was constructed for insertion of genes into the xylose isomerase region of the Deletion strain. We intended to PCR-amplify a fragment denoted "C" comprising the *xylA* promoter and about 1 kb of the *xylA* gene. However, as later described, only a smaller fragment denoted "D" comprising the *xylA* promoter and the first 250 basepairs of the *xylA* gene was succesfully amplified and cloned. The Integration plasmid comprises fragments A and D on a temperature-sensitive vector. An expression cassette was also cloned in the Integration plasmid between fragments A and D.

The temperature-sensitive Integration plasmid was transferred to the *B. licheniformis* Deletion strain and it integrated in the chromosome; subsequent excision of the temperature sensitive vector was ensured, and "Integration strains" could then be isolated which grow on minimal media with xylose as sole carbon source. Such Integration strains have restored the chromosomal *xylA* gene, by double homologous recombination via fragments A and D. In this process, the expression cassette has been integrated into the chromosome.

Plasmid constructs

PCR amplifications were performed with Ready-To-Go PCR Beads from amersham pharmacia biotech as described in the manufacturers instructions, using an annealing temperature of 55°C.

Plasmids pSJ5128 and pSJ5129:

The A fragment (*xylR* promoter and part of the *xylR* gene) was amplified from *Bacillus licheniformis* PL1980 chromosomal DNA using primers:

#183235; [*Hin*dIII ←Z80222 1242-1261→] (SEQ ID 1):
5'-GACTAAGCTTCTGCATAGTGAGAGAAGACG

#183234: [*Eco*RI; *Bgl*II; *Not*I; *Mlu*I; *Sal*I; *Sca*I ←Z80222 2137-2113→] (SEQ ID 2):
5'-GACTGAATTCAGATCTGCGGCCGCACGCGTGTCGACAGTACTGAAATAGAGGAAAAAATAAGTTTTC

The PCR fragment was digested with *Eco*RI and *Hin*dIII and purified, then ligated to *Eco*RI and *Hin*dIII digested pUC19. The ligation mixture was transformed by electroporation into *E. coli* SJ2, and transformants were selected for ampicillin resistance (200 μg/ml). The PCR-fragments of three such ampicillin resistant transformants were sequenced and all were found to be correct. Two clones designated SJ5128 (SJ2/pSJ5128) and SJ5129 (SJ2/pSJ5129) were kept.

Plasmids pSJ5124, pSJ5125:

The B fragment (an internal part of *xylA*, missing the promoter and the first 70 basepairs of the coding region), was amplified from *B. licheniformis* PL1980 chromosomal DNA using primers:

#183230 [*Eco*RI ←Z80222 3328-3306→] (SEQ ID 3):
5'-GACTGAATTCCGTATCCATTCCTGCGATATGAG

#183227 [*Bam*HI; *Bgl*II ←Z80222 2318-2342→] (SEQ ID 4):
5'-GACTGGATCCAGATCTTATTACAACCCTGATGAATTTGTCG

The PCR fragment was digested with *Eco*RI and *Bam*HI, and purified, then ligated to *Eco*RI + *Bam*HI digested pUC19 and transformed by electroporation into *E. coli* SJ2. Transformants were selected for ampicillin resistance (200 μg/ml). Two clones were correct as confirmed by DNA sequencing, and were kept as SJ5124 (SJ2/pSJ5124) and SJ5125 (SJ2/pSJ5125).

Plasmid pSJ5130:

The C fragment (comprising the *xylA* promoter and about 1 kb of the *xylA* gene) was PCR amplified from *B. licheniformis* PL1980 chromosomal DNA using primers:

#183230 (see above, SEQ ID 3)

#183229 [*Bam*HI; *Bgl*II; *Nhe*I; *Cla*I; *Sac*II ←Z80222 2131-2156→] (SEQ ID 5):
5'-GACTGGATCCAGATCTGCTAGCATCGATCCGCGGCTATTTCCATTGAAAGCGATTAATTG

The PCR fragment was digested with *Eco*RI and *Bam*HI and purified, then ligated to *Eco*RI and *Bam*HI digested pUC19 and transformed by electroporation, into *E. coli* SJ2. Transformants were selected for ampicillin resistance (200 μg/ml). One clone, comprising the full-length PCR fragment, was found to have a single basepair deletion in the promoter region, between the -35 and -10 sequences. This transformant was kept as SJ5130 (SJ2/pSJ5130).

Plasmid pSJ5131:

This plasmid was constructed as pSJ5130, above, but turned out to contain a 400 basepair PCR fragment only (the D fragment), comprising the *xylA* promoter and the first 250 basepairs of the *xylA* coding sequence. DNA sequencing confirmed that the no sequence errors were present in the fragment. The transformant was kept as SJ5131 (SJ2/pSJ5131).

Plasmids pSJ5197, pSJ5198:

These plasmids comprise the A (*xylR*) fragment on a temperature-sensitive, mobilizable vector. They were constructed by ligating the 0.9 kb *Bgl*II-*Hin*dIII fragment from pSJ5129 to the 5.4 kb *Bgl*II-*Hin*dIII fragment from pSJ2739, and transforming *B. subtilis* DN1885 competent cells with the ligation mix followed by selecting for erythromycin resistance (5 μg/ml). Two clones were kept, SJ5197 (DN1885/pSJ5197) and SJ5198 (DN1885/pSJ5198).

Plasmids pSJ5211, pSJ5212:

These plasmids contain a *res-spc-res* cassette inserted next to the B fragment. They were constructed by ligating the 1.5 kb *Bcl*I-*Bam*HI fragment from pSJ3358 into the *Bgl*II site of pSJ5124, and transforming the ligation mix into *E. coli* SJ2 and selecting for ampicillin resistance (200 μg/ml) and spectinomycin resistance (120 μg/ml) resistance. Two clones were kept, wherein the *res-spc-res* cassette was inserted in either of the possible orientations, SJ5211 (SJ2/pSJ5211) and SJ5212 (SJ2/pSJ5212).

The Deletion plasmid

Plasmid pSJ5218:

This plasmid contains the *res-spc-res* cassette flanked by the A and B fragments. It was constructed by ligating the 2.5 kb *Eco*RI-*Bam*HI fragment from pSJ5211 to the 5.3 kb *Eco*RI-*Bgl*II fragment from pSJ5197, and transforming the ligation mix into *B. subtilis* DN1885 and selecting for erythromycin (5 μg/ml) and spectinomycin resistance (120 μg/ml) resistance at 30°C. One transformant, SJ5218 (DN1885/pSJ5218) was kept.

The Integration plasmids

Plasmids pSJ5247, pSJ5248:

These plasmids comprise the short 400 basepairs D fragment (*PxylA-xylA*) as well as the A fragment (*xylR*) on a temperature-sensitive, mobilizable vector. They were made by ligating the 0.4 kb *Bgl*II-*Eco*RI fragment from pSJ5131 to the 5.3 kb *Bgl*II-*Eco*RI fragment from pSJ5197, and transforming the ligation mix into *B. subtilis* DN1885 and selecting for erythromycin resistance (5 μg/ml) at 30°C. Two transformants, SJ5247 (DN1885/pSJ5247) and SJ5248 (DN1885/pSJ5248) were kept.

Construction of strains with chromosomal *xylA* deletions.

The deletion plasmid pSJ5218 was transformed into competent cells of the *B. subtilis* conjugation donor strain PP289-5 (which contains a chromosomal *dal*-deletion, and plasmids pBC16 and pLS20), transformants were selected for resistance to spectinomycin (120 μg/ml), erythromycin (5 μg/ml) and tetracycline (5 μg/ml) on plates with D-alanine (100 μg/ml) at 30°C. Two transformants were kept, SJ5219 and SJ5220.

The two-copy *B. licheniformis* alpha-amylase strain SJ4671, described in WO 99/41358 was used as recipient in conjugations.

Donor strains SJ5219 and SJ5220 were grown overnight at 30°C on LBPSG plates (LB plates with phosphate (0.01 M K_3PO_4), glucose (0.4 %), and starch (0.5 %)) supplemented with D-alanine (100 μg/ml), spectinomycin (120 μg/ml), erythromycin (5 μg/ml) and tetracycline (5 μg/ml). The recipient strain was grown overnight on LBPSG plates.

An inoculation needle loopful of donor and recipient were mixed on the surface of a LBPSG plate with D-alanine (100 μg/ml), and incubated at 30°C for 5 hours. This plate was then replicated onto LBPSG with erythromycin (5 μg/ml) and spectinomycin (120 μg/ml), and incubation was at 30°C for 2 days. These four conjugations resulted in between 13 and 25 transconjugants.

Tetracycline-sensitive (indicating absence of pBC16) transconjugants were reisolated on LBPSG with erythromycin (5 μg/ml) and spectinomycin (120

μg/ml) at 50°C, incubated overnight, and single colonies from the 50°C plates were inoculated into 10 ml TY liquid cultures and incubated with shaking at 26°C for 3 days. Aliquots were then transferred into fresh 10 ml TY cultures and incubation proceeded overnight at 30°C. The cultures were plated on LBPSG with 120 μg/ml spectinomycin, after overnight incubation at 30°C these plates were replica plated onto spectinomycin and erythromycin, respectively, and erythromycin sensitive, spectinomycin resistant isolates were obtained from all strain conjugations.

The following strains, containing the chromosomal *xylA* promoter and the first 70 basepairs of the *xylA* coding sequence replaced by the *res-spc-res* cassette, were kept:

SJ5231: SJ4671 recipient, SJ5219 donor.
SJ5232: SJ4671 recipient, SJ5220 donor.

Strain phenotypes were assayed on TSS minimal medium agar plates, prepared as follows. 400 ml H_2O and 10 g agar is autoclaved at 121°C for 20 minutes, and allowed to cool to 60°C. The following sterile solutions are added:

1 M Tris pH 7.5	25 ml
2 % $FeCl_3.6H_2O$	1 ml
2 % trisodium citrate dihydrate	1 ml
1 M K_2HPO_4	1.25 ml
10 % $MgSO_4.7H_2O$	1 ml
10 % glutamine	10 ml; and
20 % glucose	12.5 ml; or
15 % xylose	16.7 ml

Bacillus licheniformis SJ4671 grows well on both glucose and xylose TSS plates, forming brownish coloured colonies.

The *xylA* deletion strains SJ5231-SJ5232 grow well on glucose TSS plates, but only a very thin, transparent growth is formed on the TSS xylose plates, even after prolonged incubation. These strains are clearly unable to use xylose as the sole carbon source.

Directed and selectable integration into the *xyl* region.

Integration plasmid pSJ5247 (containing the D and A fragments), and as a negative control pSJ5198 (containing only the A fragment) were transformed into competent cells of the *B. subtilis* conjugation donor strain

PP289-5 (which contains a chromosomal *dal*-deletion, and plasmids pBC16 and pLS20), transformants were selected for resistance to erythromycin (5 μg/ml) and tetracycline (5 μg/ml) on plates with D-alanine (100 μg/ml) at 30°C. Transformants kept were:

SJ5255: PP289-5/pSJ5198.
SJ5257: PP289-5/pSJ5248.

Donor strains SJ5255 and SJ5257 were used in conjugations to recipient SJ5231. Selection of transconjugants was on erythromycin (5 μg/ml), at 30°C. Transconjugants were streaked on TSS plates with xylose, at 50°C. In parallel, SJ5221 was streaked as a xylose isomerase positive control strain (also at 50°C).

After overnight incubation, all strains had formed a very thin, transparent growth. The control, however, was better growing and colonies were brownish.

After another day of incubation at 50°C, some brownish colonies were coming up on the background of thin, transparent growth, in transconjugants derived from SJ5257, i.e. the strain containing the Integration plasmid with the *PxylA-xylA* fragment (D). These colonies were steadily growing, and further colonies were coming up, during subsequent days of continued incubation at 50°C.

No brownish colonies (and no further growth than the thin, transparent growth seen after the first overnight incubation) were observed from transconjugants derived from SJ5255 (the negative control, unable to restore the chromosomal *xylA* gene).

Directed integration of an alpha-amylase gene into the *xyl* region.

Construction of an *amyL* containing integration plasmid

Plasmids pSJ5291 and pSJ5292 were constructed from the integration vector plasmid pSJ5247 by digestion of this plasmid with *Bgl*II, and insertion of the 1.9 kb *amyL* containing *Bgl*II-*Bcl*I fragment from pSJ4457 (described in WO 99/41358). The ligation mixture was transformed into *B. subtilis* DN1885 and two transformants were kept as SJ5291 and SJ5292.

Construction of conjugative donor strains, transfer to *B. licheniformis* hosts, and chromosomal integration

Plasmids pSJ5291 and pSJ5292 were transformed into competent cells of the *B. subtilis* conjugation donor strain PP289-5 (which contains a chromosomal *dal*-deletion, and plasmids pBC16 and pLS20), transformants were

selected for resistance to erythromycin (5 μg/ml) and tetracycline (5 μg/ml) on plates with D-alanine (100 μg/ml) at 30°C.

Transformants kept were SJ5293 (PP289-5/pSJ5291) and SJ5294 (PP289-5/pSJ5292). These two strains were used as donors in conjugations to xylose isomerase deletion strains SJ5231 and SJ5232. Transconjugants were selected on LBPGA plates with erythromycin (5 μg/ml), and one or two tetracyclin-sensitive transconjugants from each conjugation were streaked on a TSS-xylose plate which was incubated at 50°C. After two days incubation, well-growing colonies were inoculated into liquid TY medium (10 ml) without antibiotics, and these cultures were incubated with shaking at 30°C. After overnight incubation, 100 μl from each culture were transferred into new 10 ml TY cultures, and incubation repeated. This procedure was repeated another two times, and in addition the cultures were plated on TSS-xylose plates at 30°C. After about a week, all plates were replicaplated onto TSS-xylose as well as LBPSG with erythromycin (5 μg/ml). The following day, putative Em-sensitive strains were restreaked on the same plate types.
The following Em sensitive strains, which all grow well on TSS-xylose plates, were kept:

SJ5308 (from conjugation donor SJ5293, host SJ5231)
SJ5309 (from conjugation donor SJ5293, host SJ5231)
SJ5310 (from conjugation donor SJ5293, host SJ5232)
SJ5315 (from conjugation donor SJ5294, host SJ5231)

Southern analysis.

The two-copy *amyL* strain SJ4671, and strains SJ5308, SJ5309, SJ5310 and SJ5315, were grown overnight in TY-glucose, and chromosomal DNA was extracted. The chromosomal DNA was digested with *Hin*dIII, fragments separated by agarose gel electrophoresis, transferred to Immobilon-N® filters (Millipore®) and hybridised to a biotinylated probe based on HindIII digested pSJ5292 (using NEBlot Photope Kit and Photope Detection Kit 6K).

In the two-copy strain, the two *amyL* gene copies reside on a ~10 kb *Hin*dIII fragment. In addition, an ~2.8 kb fragment is hybridizing, which is due to hybridization to the *xyl* region. In the four strains with insertions of a third *amyL* gene into the xylose gene region, the ~2.8 kb fragment is missing and has been replaced by a fragment of ~4.6 kb. This is entirely as expected upon integration of the *amyL* gene into the xylose gene region. The ~10 kb fragment due to the two-copy insertion is retained.
In conclusion, the southern analysis shows that strains SJ5308. SJ5309, SJ5310 and SJ5315 have a correctly inserted third *amyL* gene copy in their chromosome.

Shake flask evaluation.

Strains with the *amyL* gene integrated in the xylose isomerase region, as well as several control strains, were inoculated into 100 ml BPX medium in shake flasks and incubated at 37°C with shaking at 300 rpm for 7 days. Alpha-amylase activity in the culture broth was determinated by the Phadebas assay:

Strain	Relative alpha-amylase Units/ml
SJ4270 (one copy *amyL* strain)	100
SJ4671 (two copy *amyL* strain)	161
SJ5231 (two copy *amyL* strain with *xylA* gene deletion)	148
SJ5308 (three-copy *amyL* strain)	200
SJ5309 (three-copy *amyL* strain)	245
SJ5310 (three-copy *amyL* strain)	200
SJ5315 (three-copy *amyL* strain)	200

Aliquots from each shake flask were plated on amylase indicator plates. All colonies were amylase positive. Four single colonies from each of SJ4671, SJ5309 and SJ5315 were inoculated into fresh BPX shake flasks, which were cultured as above. Alpha-amylase activity in the culture broth was determinated by the Phadebas assay:

Strain	Relative alpha-amylase Units/ml
SJ4671 (two copy *amyL* 1 strain)	100
SJ4671	102
SJ4671	88
SJ4671	84
SJ5309 (three-copy *amyL* strain)	**149**
SJ5309	**141**
SJ5309	**135**
SJ5309	**149**
SJ5315 (three-copy *amyL* strain)	**135**
SJ5315	**147**
SJ5315	**159**
SJ5315	**153**

Under these shake flask conditions, the three copy *amyL* strains (bold) seem to produce about 50% more alpha-amylase than the two-copy strain.

Example 2

A strain of *Bacillus licheniformis* having two stably integrated *amyL* gene copies in its chromosome, inserted in opposite relative orientations in the region of the *B. licheniformis* alpha-amylase gene, *amyL*, has been described in WO 99/41358, as SJ4671. A third copy of the *amyL* gene was inserted in *xylRA* as described above

This describes the insertion into this three-copy strain of a fourth *amyL* gene copy by selectable, directed integration into another region of the *B. licheniformis* chromosome.

Gluconat deletion/integration outline (Figure 2)

The sequence region of the *Bacillus lichenformis* gluconate operon comprising the *gntR*, *gntK*, *gntP*, *gntZ* genes for utilization of gluconate is available in Genbank/EMBL with accession number D31631. The region can be schematically drawn as shown in figure 2.

A deletion was introduced by cloning, on a temperature-sensitive plasmid, the PCR amplified fragments denoted as "A" (containing part of the *gntK* and part of the *gntP* gene) and "B" (containing an internal fragment of *gntZ*). As a help in the selection of deletion strains, a kanamycine resistance gene flanked by resolvase sites was introduced between fragments "A" and "B", resulting in the plasmid denoted "Deletion plasmid" in figure 2. This kanamycine resistance gene could later be removed by resolvase-mediated site-specific recombination, as described in WO 96/23073.

The deletion was transferred to the chromosome of target strains by double homologous recombination via fragments "A" and "B", mediated by integration and excision of the temperature-sensitive plasmid. The result was the strain, labelled "Deletion strain" in figure 2. This strain is unable to grow on minimal media with gluconate as sole carbon source.

Plasmid constructs

To construct an Integration plasmid to be used for gene insertions, the PCR fragment "C" was amplified. This fragment contained an internal fragment *of gntP* of about 1 Kb. The Integration plasmid consists of fragments "B" and "C" on a temperature-sensitive vector. The expression cassette destined for integration is cloned between "B" and "C". Upon transfer to the *B. licheniformis* Deletion strain and integration and

excision of the temperature-sensitive vector, strains could be isolated which grew on minimal media with gluconate as sole carbon source. Such strains had restored the chromosomal *gntP* gene by double homologous recombination via fragments "B" and "C". In this process, the expression cassette was integrated into the chromosome resulting in the "Integration strain" of figure 2.

PCR amplifications were performed with Ready-To-Go PCR Beads from amersham pharmacia biotech as described in the manufacturers instructions, using an annealing temperature of 55°C.

The Deletion Plasmids pMOL1789 and pMOL1790:

The "B" fragment (containing the internal part of the *gntZ*) was amplified from chromosomal DNA from *Bacillus licheniformis* using primers

#187338 [*Ava*I ←D31631 4903-4922→] (SEQ ID 6):
5'-TATTTCCCGAGATTCTGTTATCGACTCGCTC

#187339 [*Eag*I ←D31631 5553-5538→] (SEQ ID 7):
5'-GTTTTCGGCCGCTGTCCGTTCGTCTTT

The fragment was digested with *Ava*I + *Eag*I, ligated to *Ava*I + *Eag*I digested pMOL1642, and the ligation mixture transformed, by transformation, into *B. subtilis* PL1801 selecting for erythromycin resistance (5 μg/ml). The insert on three clones was sequenced, and all found to be correct. MOL1789 (JA578 (*repF*⁺)/pMOL1789) and MOL1790 (JA578/pMOL1790) were kept. The endpoint of the "B" fragment relative to *gntZ* is shown in fig. 2.

Plasmids pMOL1820 and pMOL1821:

The "A" fragment (containing part of the *gntK* and part of the *gntP* gene), was amplified from chromosomal DNA of *Bacillus licheniformis* using primers

#184733 [←D31631 3738-3712→] (SEQ ID 8):
5'-GTGTGACGGATAAGGCCGCCGTCATTG

#184788 [←D31631 3041-3068→] (SEQ ID 9):
5'-CTCTTGTCTCGGAGCCTGCATTTTGGGG

The fragment was digested with *Cla*I + *Eco*RI, ligated to *Eco*RI + *Cla*I digested pMOL1789, and transformed, by transformation, into *B. subtilis* PL1801 selecting for erythromycin resistance (5 μg/ml). The insert on three

clones was sequenced, and all found to be correct. MOL1820 (JA578/pMOL1820) and MOL1821 (JA578/pMOL1821) were kept. The endpoint of the "A" fragment relative to *gntZ* is shown in fig. 2.

The Integration plasmids pMOL1912 and pMOL1913:

These plasmids contain a short C-terminal part of *gntK* and the entire open reading frame of *gntP* (the "C" fragment) on a temperature-sensitive, mobilizable vector. They were made by ligating a 0.9 kb fragment amplified from chromosomal DNA of *Bacillus licheniformis* using primers:

#B1656D07 [←D31631 3617-3642→] (SEQ ID 10):
5'-AGCATTATTCTTCGAAGTCGCATTGG

#B1659F03 [*Bgl*II←D31631 4637-4602→] (SEQ ID 11):
5'-TTAAGATCTTTTTTATACAAATAGGCTTAACAATAAAGTAAATCC

The fragment was digested with *Bgl*II + *Eco*RI, ligated to *Bgl*II + *Eco*RI digested pMOL1820, and the ligation mixture transformed, by transformation, into *B. subtilis* PL1801 selecting for erythromycin resistance (5 μg/ml). The insert on three clones was sequenced, and all found to be correct. MOL1912 (PL1801/pMOL1789) and MOL1913 (PL1801/pMOL1913) were kept. The endpoint of the "C" fragment relative to *gntZ* is shown in fig. 2.

These plasmids were found to express functional GntP even if they do not have a promoter sequence directly upstream of the *gntP* gene. In order to enable directed integration in the *gntP* region by selecting for growth on gluconate it was necessary to delete part of the N- terminal sequence of the *gntP* gene on the integration plasmid pMOL1912.

Plasmids pMOL1972 and pMOL1973:

These plasmids are Deletion derivatives of pMOL1912 which contain the entire *gntP* gene except for the first 158 bp coding for 53 amino acids of the N-terminal. The plasmid pMOL1912 was digested with *Stu*I + *Eco*RV and re-ligated. The ligation mixture was transformed, by competence, into *B. subtilis* PL1801 selecting for erythromycin resistance (5 μg/ml). The deletion was verified by restriction digest. MOL1972 (PL1801/pMOL1972) and MOL1973 (PL1801/pMOL1973) were kept.

These plasmids do not support growth on TSS gluconate plates when introduced as free plasmids in a *gntP* deleted background.

Construction of strains with chromosomal *gntP* d letions

The Deletion plasmid pMOL1920 was transformed into competent cells of the *B. subtilis* conjugation donor strain PP289-5 (which contains a chromosomal *dal*-deletion, and plasmids pBC16 and pLS20), selecting resistance to kanamycine (10 μg/ml), erythromycin (5 μg/ml) and tetracycline (5 μg/ml) on plates with D-alanine (100 μg/ml) at 30°C. Two transformants were kept, MOL1822 and MOL1823.

The two-copy *B. licheniformis* alpha-amylase strain SJ4671, described in WO 99/41358 was used as recipient in conjugations.

Donor strains MOL1822 and MOL1823.were grown overnight at 30°C on LBPSG plates (LB plates with phosphate (0.01 M K_3PO_4), glucose (0.4 %), and starch (0.5 %)) supplemented with D-alanine (100 μg/ml), kanamycine (10 μg/ml), erythromycin (5 μg/ml) and tetracycline (5 μg/ml). The recipient strain was grown overnight on LBPSG plates.

A loopful of donor and recipient were mixed on the surface of a LBPSG plate with D-alanine (100 μg/ml), and incubated at 30°C for 5 hours. This plate was then replicated onto LBPSG with erythromycin (5 μg/ml) and kanamycine (10 μg/ml), and incubation was at 30°C for 2 days. These four conjugations resulted in between 25 and 50 transconjugants.

Tetracycline-sensitive (indicating absence of pBC16) transconjugants were reisolated on LBPSG with erythromycin (5 μg/ml) and kanamycine (10 μg/ml) at 50°C, incubated overnight, and single colonies from the 50°C plates were inoculated into 10 ml TY liquid cultures and incubated with shaking at 26°C for 3 days, then aliquots were transferred into fresh 10 ml TY cultures and incubation continued overnight at 30°C. The cultures were then plated on LBPSG with 10 μg/ml kanamycine, after overnight incubation at 30°C these plates were replica plated onto kanamycine and erythromycin, respectively, and erythromycin sensitive, kanamycine resistant isolates were obtained from all strain combinations. The following strains, where part of the *gntP* gene coding for the C-terminal was replaced by the *res-kana-res* cassette, were kept:

MOL1871: SJ4671 recipient, MOL1822 donor.
MOL1872: SJ4671 recipient, MOL1823 donor.

Strain phenotypes were assayed on TSS minimal medium agar plates, prepared as follows:

400 ml H_2O is added 10 g agar and is autoclaved at 121°C for 20 minutes, and allowed to cool to 60°C. The following sterile solutions are added:

1 M Tris pH 7.5	25 ml

2 % $FeCl_3.6H_2O$	1 ml
2 % trisodium citrate dihydrate	1 ml
1 M K_2HPO_4	1.25 ml
10 % $MgSO_4.7H_2O$	1 ml
10 % glutamine	10 ml, and
20 % glucose	12.5 ml, or
15 % gluconate	16.7 ml

Bacillus licheniformis SJ4671 grows well on both glucose and gluconate TSS plates, forming brownish coloured colonies. The *gntP* Deletion strains MOL1871 and MOL1872 grow well on glucose TSS plates, but only a very thin, transparent growth is formed on the TSS gluconate plates, even after prolonged incubation. These strains are clearly unable to use gluconate as the sole carbon source.

The same *gntP* deletion procedure is performed on the three copy strain SJ5309 described earlier to prepare for integration of a fourth copy of the amylase expression cassette.

Directed and selectable integration into the *gnt* region

Integration plasmid pMOL1972 (containing the "B" and "C" fragments), and as a negative control pMOL1789 (containing only the "B" fragment), were transformed into competent cells of the *B. subtilis* conjugation donor strain PP289-5 (which contains a chromosomal *dal*-deletion, and plasmids pBC16 and pLS20), selecting resistance to erythromycin (5 μg/ml) and tetracycline (5 μg/ml) on plates with D-alanine (100 μg/ml) at 30°C. Transformants kept were:

MOL1974: PP289-5/pMOL1972.
MOL1975: PP289-5/pMOL1973.

Donor strains MOL1974 and MOL1975 were used in conjugations to recipient MOL1871 and MOL1872. Selection of transconjugants was on erythromycin (5 μg/ml), at 30°C. Transconjugants were streaked on TSS plates with gluconate, at 50°C. In parallel, SJ4671 was streaked as a gluconate positive control strain (also at 50°C).

After overnight incubation, all strains had formed a very thin, transparent growth. The control, however, was better growing and colonies were brownish. After another day of incubation at 50°C, some brownish colonies were coming up on the background of thin, transparent growth, in transconjugants derived from MOL1871 and MOL1872. These colonies were

steadily growing, and further colonies appeared, during subsequent days of continued incubation at 50°C.

No colonies were observed from the *gntP* deleted strains MOL1871 and MOL1872.

Directed integration of an alpha-amylase gene into the *gnt* region

Construction of an *amyL* containing Integration plasmid.

The following is a construction plan for integrating an expression cassette with the alpha-amylase gene in the *gnt* region making use of the selection principle described above. The integration plasmid pMOL1972 is digested with *Bgl*II, and a 1.9 kb *Bgl*II-*Bcl*I fragment containing *amyL* from pSJ4457 (described in WO 99/41358) is inserted by ligation. The ligation mixture is then transformed into *B. subtilis* DN1885 and transformants selected on LBPSG plates with erythromycin (5 μg/ml) are verified by restriction digestion of plasmid DNA.

Conjugative donor strains, transfer to *B. licheniformis*, and chromosomal integration.

The Integration plasmid with the expression cassette is transformed into competent cells of the *B. subtilis* conjugation donor strain PP289-5 (which contains a chromosomal *dal*-deletion, and plasmids pBC16 and pLS20), selecting resistance to erythromycin (5 μg/ml) and tetracycline (5 μg/ml) on plates with D-alanine (100 μg/ml) at 30°C.

Transformants comprising the Integration plasmid with the expression cassette are preserved and used as donors in conjugations with a *gntP* Deletion recipient of the three-copy strain SJ5309, which in turn was constructed as described for the Deletion strains MOL1871 and MOL1872 described above.

Transconjugants are selected on LBPGA plates with erythromycin (5 μg/ml), and one or two tetracyclin-sensitive transconjugants from each conjugation is streaked on a TSS-gluconate plate which is incubated at 50°C. After two days incubation, well-growing colonies are inoculated into liquid TY medium (10 ml) without antibiotics, and these cultures are incubated with shaking at 30°C. After overnight incubation, 100 μl from each culture is transferred into new 10 ml TY cultures, and incubated. This procedure is repeated twice, and in addition the cultures are plated on TSS-gluconate plates at 30°C.

After about a week, all plates are replica-plated onto TSS-gluconate as well as LBPSG with erythromycin (5 μg/ml) and incubated. The following day putative Em-sensitive strains are restreaked on the same plate types

As for integration in the xylose region described earlier, Southern analysis and shake flask evaluation is performed to verify the site of integration in the *gnt* region of the alpha-amylase expression cassette and the increased yield from this four copy strain.

Example 3

Bacillus licheniformis SJ4671 (WO 99/41358) comprises two stably integrated *amyL* gene copies in its chromosome, inserted in opposite relative orientations in the region of the *B. licheniformis* alpha-amylase gene, *amyL*. The following example describes the insertion into this strain of a third *amyL* gene copy by selectable, directed integration into another region of the *B. licheniformis* chromosome.

D-alanine racemase deletion/integration outline

The DNA sequence of the *Bacillus lichenformis* D-alanine racemase region was determined in this work and is shown in positions 1303 to 2469 in SEQ ID 12. A plasmid denoted "Dal-Deletion plasmid" was constructed by cloning one 2281 bp PCR amplified fragment from the D-alanine racemase region of *Bacillus lichenformis* on a temperature-sensitive parent plasmid. The PCR 2281 bp fragment was denoted "A", wherein A comprises the sequence from 245 basepairs upstream of the ATG start codon of the *dal* gene to 867 basepairs downstream of the *dal* gene.

A deletion of 586 basepairs of the C-terminal part of the *dal* gene on the cloned fragment A was done resulting in a plasmid containing the fragments "B" and "C" as shown below. A spectinomycin resistance gene flanked by resolvase (*res*) sites was introduced between fragments "B" and "C" on the plasmid. This spectinomycin resistance gene could later be removed by resolvase-mediated site-specific recombination.

The D-alanine racemase deletion was transferred from the Dal-Deletion plasmid to the chromosome of a *Bacillus* target strain by double homologous recombination via fragments "B" and "C", mediated by integration and excision of the temperature-sensitive Dal-Deletion plasmid. The resulting strain was denoted "Dal-Deletion strain". This strain was unable to grow on media without D-alanine.

An Integration plasmid was constructed for insertion of genes into the D-alanine region of the Deletion strain. We intended to PCR-amplify a fragment denoted "D" comprising 1117 basepairs of the *dal* gene starting from 41 basepairs downstream of the ATG start codon. The promoter region was substituted with the T1 and T2 terminators from the 3'-terminal sequence of the Escherichia coli *rrn*B ribosome RNA operon (EMBL/e09023: basepair 197-295).

The Integration plasmid comprises fragments D and C on a temperature-sensitive vector. An expression cassette destined for integration was cloned between the fragments D and C. Upon transfer to the *B. licheniformis* deletion strain, integration, and excision of the temperature-sensitive vector, strains could be isolated which grow on media without D-alanine. Such "Integration strains" have restored the chromosomal *dal* gene, by double homologous recombination via fragments D and C. In this process, the expression cassette was integrated into the chromosome.

Plasmid constructs

PCR amplifications were performed with Ready-To-Go PCR Beads from amersham pharmacia biotech as described in the manufacturers instructions, using an annealing temperature of 55°C.

Plasmids pJA744:

The A fragment (*dal*-region) was amplified from *Bacillus licheniformis* SJ4671 chromosomal DNA using primers:

#148779; [Upstream of a *Sph*I site in the *dal* region] (SEQ ID 14):

5'-GATGAACTTCTGATGGTTGC

#148780: [*Bam*HI < *dal*] (SEQ ID 15):

5'-AAAGGATCCCCCTGACTACATCTGGC

The PCR fragment was digested with *Sph*I and *Bam*HI and purified, then ligated to *Sph*I and *Bam*HI digested pPL2438. Transforming *B. subtilis* JA691 (*repF*$^+$, *dal*$^-$) competent cells with the ligation mix followed by selecting for kanamycin resistance (10 µg/ml). Correct clones could complement the JA691 *dal* phenotype.

Plasmid pJA770:

This plasmid contains a *res-spc-res* cassette inserted between the B and C fragments. It was constructed by ligating the 1.5 kb *Bcl*I-*Bam*HI fragment from pSJ3358 into the *Bcl*I - *Bcl*I sites of pJA744. Transforming *B. subtilis* JA691 competent cells with the ligation mix followed by selecting for kanamycin resistance (10 µg/ml) and spectinomycin resistance (120 µg/ml). Orientation of the spectinomycin resistance gene was could be determined by cutting with *Bcl*I and *Bam*HI.

Dal Deletion plasmid

Plasmid pJA851:

A fragment (comprising the *ermC* gene and the replication origin of pE194) was PCR amplified from pSJ2739 plasmid DNA using primers:

#170046 [*Not*I; < *ermC* gene and the replication origin of pE194>] (SEQ ID 16)
5'-AAAGCGGCCGCGAGACTGTGACGGATGAATTGAAAAAGC

#170047 [*Eco*RI; ← *ermC* gene and the replication origin of pE194→] (SEQ ID 17):
5'-AAAGAATTCGTGAAATCAGCTGGACTAAAAGG

The PCR fragment was digested with *Eco*RI and *Not*I and purified, then ligated to *Eco*RI and *Not*I digested pJA770. Transforming *B. subtilis* JA691 competent cells with the ligation mix followed by selecting for erythromycin resistance (5 μg/ml) and spectinomycin resistance (120 μg/ml).

Plasmid PJA748:

A fragment (comprising the *dal* gene without the promotor region) was PCR amplified from Bacillus licheniformis SJ4671 DNA using primers:

#150506 [*Bam*HI; < *dal* gene] (SEQ ID 18)
5'-AAAGGATCCCGCAAGCAAAGTTGTTTTTCCGC

#150507 [*Kpn*I; <- *dal* gene] (SEQ ID 19):
5'-AAAGGTACCGAAAGACATGGGCCGAAATCG

The PCR fragment was digested with *Kpn*I and *Bam*HI and purified, then ligated to *Kpn*I and *Bam*HI digested pPL2438. Transforming *B. subtilis* JA691 competent cells with the ligation mix followed by selecting for kanamycin resistance (10 μg/ml).

Plasmids pJA762:

A fragment (comprising the T_1 and T_2 Terminators from the *E.coli rrnB* terminal sequence EMBL[e09023] from basepair 197 to 295) was PCR amplified from *Escherichia coli* SJ2 DNA using primers:

#158089 [*Kpn*I; < T_1 and T_2 Terminators of *rrnB*] (SEQ ID 20)
5'-AAAGGTACCGGTAATGACTCTCTAGCTTGAGG

#158090 [*Cla*I; < T_1 and T_2 Terminators of *rrnB*] (SEQ ID 21):
5'-CAAATCGATCATCACCGAAACGCGGCAGGCAGC

The PCR fragment was digested with *Kpn*I and *Cla*I and purified, then ligated to *Kpn*I and *Cla*I digested pJA748. Transforming *B. subtilis* JA691 competent cells with the ligation mix followed by selecting for kanamycin resistance (10 µg/ml).

Plasmids pJA767:

A fragment (comprising the 0.7kbp DNA sequence downstream of *dal* (DFS)) was PCR amplified from *Escherichia coli* SJ2 DNA using primers:

#150508 [*Hind*III; < DFS] (SEQ ID 22)

5'-ATTAAGCTTGATATGATTATGAATGGAATGG

#150509 [*Nhe*I; < DFS] (SEQ ID 23):

5'-AAAGCTAGCATCCCCCTGACTACATCTGGC

The PCR fragment was digested with *Hind*III and *Nhe*I and purified, then ligated to *Kpn*I and *Cla*I digested pJA762. Transforming *B. subtilis* JA691 competent cells with the ligation mix followed by selecting for kanamycin resistance (10 µg/ml).

Plasmid pJA776

This plasmid contains the *amyL* cassette flanked by the D and C fragments. It was constructed by ligating the 2.8 kb *Hind*III-*Nhe*I fragment from pSJ4457 to the 4.2 kb *Bam*HI-*Hind*III fragment from pJA767, and transforming the ligation mix into *B. subtilis* JA691 competent cells followed by selecting for kanamycin resistance (10 µg/ml).

Dal Integration plasmid

Plasmid pJA1020:

This plasmid contains the *amyL* cassette flanked by the D and C fragments. Further the plasmid contains the plasmid pE194 replication origin, *repF* and the Em^r-gene. It was constructed by ligating the 2.7kb *Eco*RI-*Nhe*I fragment of pJA776 to the 3.8kb *Eco*RI-*Nhe*I fragment of pJA851, and transforming the ligation mix into *B. subtilis* JA691 competent cells followed by selecting for erythromycin resistance (5 µg/ml).

Construction of chromos mal *dal* deletions

The Deletion plasmid pJA851 was transformed into competent cells of the *B. subtilis* conjugation donor strain PP289-5 (which contains a chromosomal *dal*-deletion, and plasmids pBC16 and pLS20), and transformants were selected for resistance to spectinomycin (120 µg/ml), erythromycin (5 µg/ml), and tetracycline (5 µg/ml) on plates with D-alanine (100 µg/ml) at 30°C. Transformants were kept as JA954 and used as donor in the following conjugation experiments.

The two-copy *amyL B. licheniformis* SJ4671 (WO 99/41358) was used as recipient in the following conjugation experiments.

Donor strain JA954 were grown overnight at 30°C on LBPSG plates (LB plates with phosphate (0.01 M K_3PO_4), glucose (0.4 %), and starch (0.5 %)) supplemented with D-alanine (100 µg/ml), spectinomycin (120 µg/ml), erythromycin (5 µg/ml) and tetracycline (5 µg/ml). The recipient strain SJ4671 was grown overnight on LBPSG plates.

Approx. one loop of an inoculation needle of donor and recipient each were mixed on the surface of a LBPSG plate with D-alanine (100 µg/ml), and incubated at 30°C for 5 hours. This plate was then replicated onto LBPSG with erythromycin (5 µg/ml) and spectinomycin (120 µg/ml), and was incubated at 30°C for 2 days. These four conjugations resulted in 13 - 25 transconjugants.

Tetracycline-sensitive (indicating absence of pBC16) transconjugants were reisolated on LBPSG plates with erythromycin (5 µg/ml) and spectinomycin (120 µg/ml) at 50°C, and incubated overnight. Single colonies from the 50°C plates were inoculated into 10 ml TY liquid medium and incubated with shaking at 26°C for 3 days, whereafter aliquots were transferred into fresh 10 ml TY cultures and incubation was continued overnight at 30°C. The cultures were plated on LBPSG with 120 µg/ml spectinomycin, after overnight incubation at 30°C these plates were replica plated onto spectinomycin and erythromycin, respectively.

Erythromycin sensitive, spectinomycin resistant isolates were obtained from all strain combinations. The following straincomprising the chromosomal *dal* promoter and the first 672 basepairs of the *dal* coding sequence replaced by the *res-spc-res* cassette, was kept:

B. licheniformis JA967: SJ4671 recipient, JA954 donor.

Strain phenotypes were assayed on LBPG with 120µg spectinomycin supplemented with or without D-alanine (100 µg/ml)

Bacillus licheniformis SJ4671 grows well on both plates with or without D-alanine. The *xylA* deletion strain JA967 growth well on LBPG D-

alanine plates, but not on LBPG plates without D-alanine. These strains are clearly unable to grow without adding D-alanine to the media.

The sequence of the B. licheniformis *dal*-region (SEQ ID 12):

The *dal*-region (comprising the *ydcC* gene, a terminator, the *dal* gene and the sequence downstream of *dal* (*DFS*)) was PCR amplified from *Bacillus licheniformis* ATCC14580 chromosomal DNA using the primers:

#145507 [< *ydcC* - *dal* - *DFS* >] (SEQ ID 24):

5'-GCGTACCGTTAAAGTCGAACAGCG

#150509 [*NheI*; < *ydcC* - *dal* - *DFS* >] (SEQ ID 25):

5'-AAAGCTAGCATCCCCCTGACTACATCTGGC

Sequencing of the D-alanine encoding sequence of *Bacillus licheniformis* that is shown in positions 1303-2469 of SEQ ID 12 and a subsequent homology search in the public databases revealed that the newly isolated *dal* gene has a sequence identity of only approx. 67% with the *dal* gene of *Bacillus subtilis*, no other D-alanine racemase encoding genes show a higher homolgoy to this new *B. licheniformis dal* gene.

Claims

1. A method for constructing a cell comprising at least two copies of a gene of interest stably integrated into the chromosome in different positions, the method comprising the steps of:

a) providing a host cell comprising at least one chromosomal copy of the gene of interest;

b) altering a conditionally essential chromosomal gene(s) of the host cell whereby the gene becomes non-funtional;

c) making a DNA construct comprising:

i) an altered non-functional copy of the chromosomal gene(s) of step b); and

ii) at least one copy of the gene of interest flanked on one side by i) and on the other side by a DNA fragment homologous to a host cell DNA sequence adjacent to the gene(s) of step b); wherein a first recombination between the altered copy of i) and the altered chromomosomal gene(s) of step b) restores the chromosomal gene(s) to functionality and renders the cell selectable;

d) introducing the DNA construct into the host cell and cultivating the cell under conditions that require a functional gene(s) of step b); and

e) selecting a host cell that grows under the conditions of step d); wherein the at least one copy of the gene of interest has integrated into the host cell chromosome adjacent to the gene(s) of step b); and optionally

f) repeating steps a) to e) at least once using a different chromosomal gene(s) in step b) in each repeat.

2. The method of claim 1, wherein subsequent to step d) and prior to step e) a second recombination takes place between the DNA fragment and the homologous host cell DNA sequence.

3. The method of claim 2, where the DNA construct further comprises a marker gene which is located in the construct such that it is recombined out of the chromosome by the second recombination.

4. The method of claim 3, wherein the marker gene confers resistance to an antibiotic, preferably the antibiotic is chosen from the group consisting of chloramphenicol, kanamycin, ampillin, erythromycin, spectinomycin and tetracycline.

5. The method of claims 3 or 4, wherein a host cell is selected which grows under the conditions of claim 1 step d) and which does not contain the marker gene in the chromosome.

6. The method of any of claims 1 - 5, where the DNA construct further comprises a marker gene located between the altered copy and the DNA fragment, and wherein the marker gene is flanked by nucleotide sequences that are recognized by a specific resolvase, preferably the nucleotide sequences are *res*.

7. The method of claim 6, wherein the marker gene is excised from the chromosome by the action of a resolvase enzyme after step e) and prior to step f).

8. The method of any of claims 1 - 7, wherein the gene of interest originates from the host cell.

9. The method of any of claims 1 - 8, wherein the gene of interest encodes an enzyme, preferably an amylolytic enzyme, a lipolytic enzyme, a proteolytic enzyme, a cellulytic enzyme, an oxidoreductase or a plant cell-wall degrading enzyme, and more preferably an enzyme with an activity selected from the group consisting of aminopeptidase, amylase, amyloglucosidase, carbohydrase, carboxypeptidase, catalase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, esterase, galactosidase, beta-galactosidase, glucoamylase, glucose oxidase, glucosidase, haloperoxidase, hemicellulase, invertase, isomerase, laccase, ligase, lipase, lyase, mannosidase, oxidase, pectinase, peroxidase, phytase, phenoloxidase, polyphenoloxidase, protease, ribonuclease, transferase, transglutaminase, or xylanase.

10. The method of any of claims 1 - 9, wherein the host cell selected in step e) comprises DNA only of endogenous origin.

11. The method of any of claims 1 - 10, wherein the chromosomal gene(s) of the host cell is altered by partially deleting the gene, or by introducing mutations in the gene(s).

12. The method of any of claims 1 - 11, wherein the chromosomal gene(s) of the host cell that is altered in step b) encodes a D-alanine racemase, preferably the gene(s) is a *dal* homologue from a *Bacillus* cell, more

preferably the gene is homologous to *dal* from *Bacillus subtilis*, and most preferably the gene(s) is the *dal* gene of *Bacillus licheniformis*.

13. The method of any of claims 1 - 11, wherein the chromosomal gene(s) of the host cell that is altered in step b) encodes a D-alanine racemase and is at least 75% identical, preferably 85% identical, more preferably 95% and most preferably at least 97% identical to the *dal* sequence of *Bacillus licheniformis* shown in positions 1303 to 2469 in SEQ ID 12.

14. The method of any of claims 1 - 11, wherein the chromosomal gene(s) of the host cell that is altered in step b) is one or more genes of a xylose isomerase operon, preferably the gene(s) is homologous to the *xylR* gene or the *xylA* gene from *Bacillus subtilis*, more preferably the gene(s) is homologous to both *xylR* and *xylA*, and most preferably the gene(s) is homologous to one or more genes of the xylose isomerase operon of *Bacillus licheniformis*.

15. The method of any of claims 1 - 11, wherein the chromosomal gene(s) of the host cell that is altered in step b) encodes a galactokinase (EC 2.7.1.6), an UTP-dependent pyrophosphorylase (EC 2.7.7.10), an UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), or an UDP-galactose epimerase (EC 5.1.2.3), preferably the gene(s) encodes an UDP-galactose epimerase (EC 5.1.2.3), more preferably the gene(s) is homologous to *galE* of a *Bacillus*, and most preferably the gene is *galE* of *Bacillus licheniformis*.

16. The method of any of claims 1 - 11, wherein the chromosomal gene(s) of the host cell that is altered in step b) is one or more genes of a gluconate operon, preferably the gene(s) encodes a gluconate kinase (EC 2.7.1.12) or a gluconate permease or both, more preferably the gene(s) is one or more genes homologous to genes from *Bacillus subtilis* chosen from the group consisting of *gntR*, *gntK*, *gntP*, and *gntZ*, and most preferably the gene(s) is one or more genes of *gntR*, *gntK*, *gntP*, and *gntZ* from *Bacillus licheniformis*.

17. The method of any of claims 1 - 16, wherein the host cell is a Gram-positive bacterial cell, preferably a *Bacillus* cell, and most preferably a *Bacillus* cell chosen from the group consisting of *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus clausii*, *Bacillus coagulans*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus stearothermophilus*, *Bacillus subtilis*, and *Bacillus thuringiensis*.

18. The method of any of claims 1 - 17, wherein the DNA construct is a plasmid.

19. A DNA construct comprising:

i) an altered non-functional copy of a conditionally essential chromosomal gene(s) from a host cell, preferably the copy is partially deleted; and

ii) at least one copy of a gene of interest flanked on one side by i) and on the other side by a DNA fragment homologous to a host cell DNA sequence located on the host cell chromosome adjacent to the conditionally essential gene(s) of i).

20. The DNA construct of claim 19, wherein the chromosomal gene(s) of the host cell that is altered in i) encodes a D-alanine racemase, preferably the gene(s) is a *dal* homologue from a *Bacillus* cell, more preferably the gene is homologous to *dal* from *Bacillus subtilis*, and most preferably the gene is the *dal* gene of *Bacillus licheniformis*.

21. The DNA construct of claim 19, wherein the chromosomal gene(s) of the host cell that is altered in i) encodes a D-alanine racemase and is at least 75% identical, preferably 85% identical, more preferably 95% and most preferably at least 97% identical to the *dal* sequence of *Bacillus licheniformis* shown in positions 1303 to 2469 in SEQ ID 12.

22. The DNA construct of claim 19, wherein the chromosomal gene(s) of the host cell that is altered in i) is one or more genes of a xylose isomerase operon, preferably the gene(s) is homologous to the *xylR* gene or the *xylA* gene from *Bacillus subtilis*, more preferably the gene(s) is both a *xylR* and a *xylA* homologue, and most preferably the gene(s) is homologous to one or more genes of the xylose isomerase operon of *Bacillus licheniformis*.

23. The DNA construct of claim 19, wherein the chromosomal gene(s) of the host cell that is altered in i) encodes a galactokinase (EC 2.7.1.6), an UTP-dependent pyrophosphorylase (EC 2.7.7.10), an UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), or an UDP-galactose epimerase (EC 5.1.2.3), preferably the gene(s) encodes an UDP-galactose epimerase (EC 5.1.2.3), more preferably the gene(s) is homologous to the *galE* gene of *Bacillus subtilis*, and most preferably the gene(s) is the *galE* gene of *Bacillus licheniformis*.

24. The DNA construct of claim 19, wherein the chromosomal gene(s) of the host cell that is altered in i) is one or more genes of a gluconate operon, preferably the gene(s) encodes a gluconate kinase (EC 2.7.1.12) or a gluconate permease or both, more preferably the gene(s) is homologous to one or more genes from *Bacillus subtilis* chosen from the group consisting of *gntR, gntK, gntP,* and *gntZ,* and most preferably the gene(s) is one or more genes of *gntR, gntK, gntP,* and *gntZ* from *Bacillus licheniformis.*

25. A host cell comprising at least two copies of a gene of interest stably integrated into the chromosome, where each copy is integrated adjacent to different conditionally essential *loci,* wherein the cell is obtainable by any of the methods defined in claims 1 - 18.

26. The cell of claim 25, wherein the gene of interest is separated from the conditionally essential *locus* by no more than 1000 basepairs, preferably no more than 750 basepairs, more preferably no more than 500 basepairs, even more preferably no more than 250 basepairs, and most preferably no more than 100 basepairs.

27. The cell of claims 25 or 26, which contains only endogenous DNA.

28. The cell of any of claims 25 - 27, which is a Gram-positive bacterial cell, preferably a *Bacillus* cell, and most preferably a *Bacillus* cell chosen from the group consisting of *Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus brevis, Bacillus circulans, Bacillus clausii, Bacillus coagulans, Bacillus lautus, Bacillus lentus, Bacillus licheniformis, Bacillus megaterium, Bacillus stearothermophilus, Bacillus subtilis,* and *Bacillus thuringiensis.*

29. The cell of any of claims 25 - 27, wherein a copy of the gene of interest is integrated adjacent to a gene encoding a D-alanine racemase, preferably a gene homologous to the *dal* gene from *Bacillus subtilis,* more preferably a gene at least 75% identical to the *dal* sequence of *Bacillus licheniformis* shown in positions 1303 to 2469 in SEQ ID 12, even more preferably a gene at least 85% identical, more preferably at least 95% and most preferably at least 97% identical to the *dal* sequence of *Bacillus licheniformis* shown in positions 1303 to 2469 in SEQ ID 12

30. The cell of any of claims 25 - 27, wherein a copy of the gene of interest is integrated adjacent to a gene of a xylose isomerase operon, preferably adjacent to genes homologous to the *xylR* or *xylA* genes from

Bacillus subtilis, and most preferably adjacent to *xylR* or *xylA* from *Bacillus licheniformis*.

31. The cell of any of claims 25 - 27, wherein a copy of the gene of interest is integrated adjacent to a gene encoding a galactokinase (EC 2.7.1.6), an UTP-dependent pyrophosphorylase (EC 2.7.7.10), an UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), or an UDP-galactose epimerase (EC 5.1.2.3), preferably adjacent to a gene encoding an UDP-galactose epimerase (EC 5.1.2.3), more preferably adjacent to a gene homologous to the *galE* gene from *Bacillus subtilis*, and most preferably adjacent to *galE* from *Bacillus licheniformis*.

32. The cell of any of claims 25 - 27, wherein a copy of the gene of interest is integrated adjacent to a gene of a gluconate operon, preferably adjacent to a gene that encodes a gluconate kinase (EC 2.7.1.12) or a gluconate permease, more preferably adjacent to a gene homologous to a *Bacillus subtilis* gene chosen from the group consisting of *gntR*, *gntK*, *gntP*, and *gntZ*, and most preferably adjacent to *gntR*, *gntK*, *gntP*, or *gntZ* from *Bacillus licheniformis*.

33. The cell of any of claims 25 - 32, wherein the gene of interest encodes an enzyme, preferably an amylolytic enzyme, a lipolytic enzyme, a proteolytic enzyme, a cellulytic enzyme, an oxidoreductase or a plant cell-wall degrading enzyme, and more preferably an enzyme with an activity selected from the group consisting of aminopeptidase, amylase, amyloglucosidase, carbohydrase, carboxypeptidase, catalase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, esterase, galactosidase, beta-galactosidase, glucoamylase, glucose oxidase, glucosidase, haloperoxidase, hemicellulase, invertase, isomerase, laccase, ligase, lipase, lyase, mannosidase, oxidase, pectinase, peroxidase, phytase, phenoloxidase, polyphenoloxidase, protease, ribonuclease, transferase, transglutaminase, or xylanase.

34. The cell of any of claims 25 - 33, wherein no antibiotic markers are present.

35. A *Bacillus licheniformis* cell, wherein at least two conditionally essential genes are rendered non-functional, preferably the genes are chosen from the group consisting of *xylR*, *xylA*, *galE*, *gntR*, *gntK*, *gntP*, *gntZ*, and *dal*.

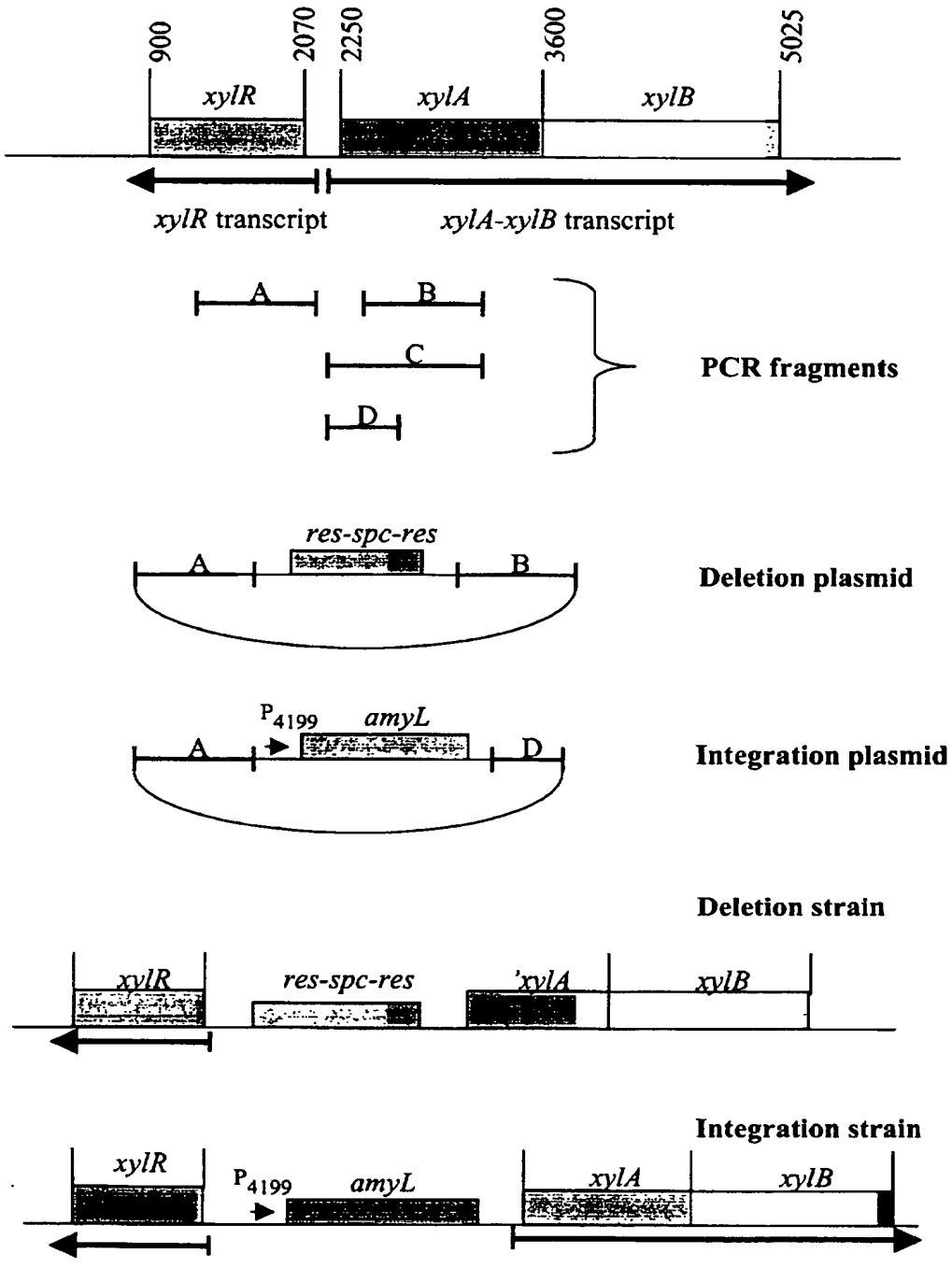
36. Use of a cell as defined in claim 35 in a method as defined in any of claims 1 - 18.

37. A cell comprising a DNA construct as defined in claims 19 - 24.

38. A process for producing an enzyme of interest, comprising cultivating a cell as defined in any of claims 25 - 34 under conditions appropriate for producing the enzyme, and optionally purifying the enzyme.

Abstract

The present invention solves the problem of integrating multiple copies of a gene of interest by homologous recombination into well defined positions adjacent to conditionally essential genes in a bacterial host strain chromosome, which already comprises at least one copy of the gene of interest in a different position.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C
D
PCR fragments
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A
B
Deletion plasmid
P4199
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A
D
Integration plasmid
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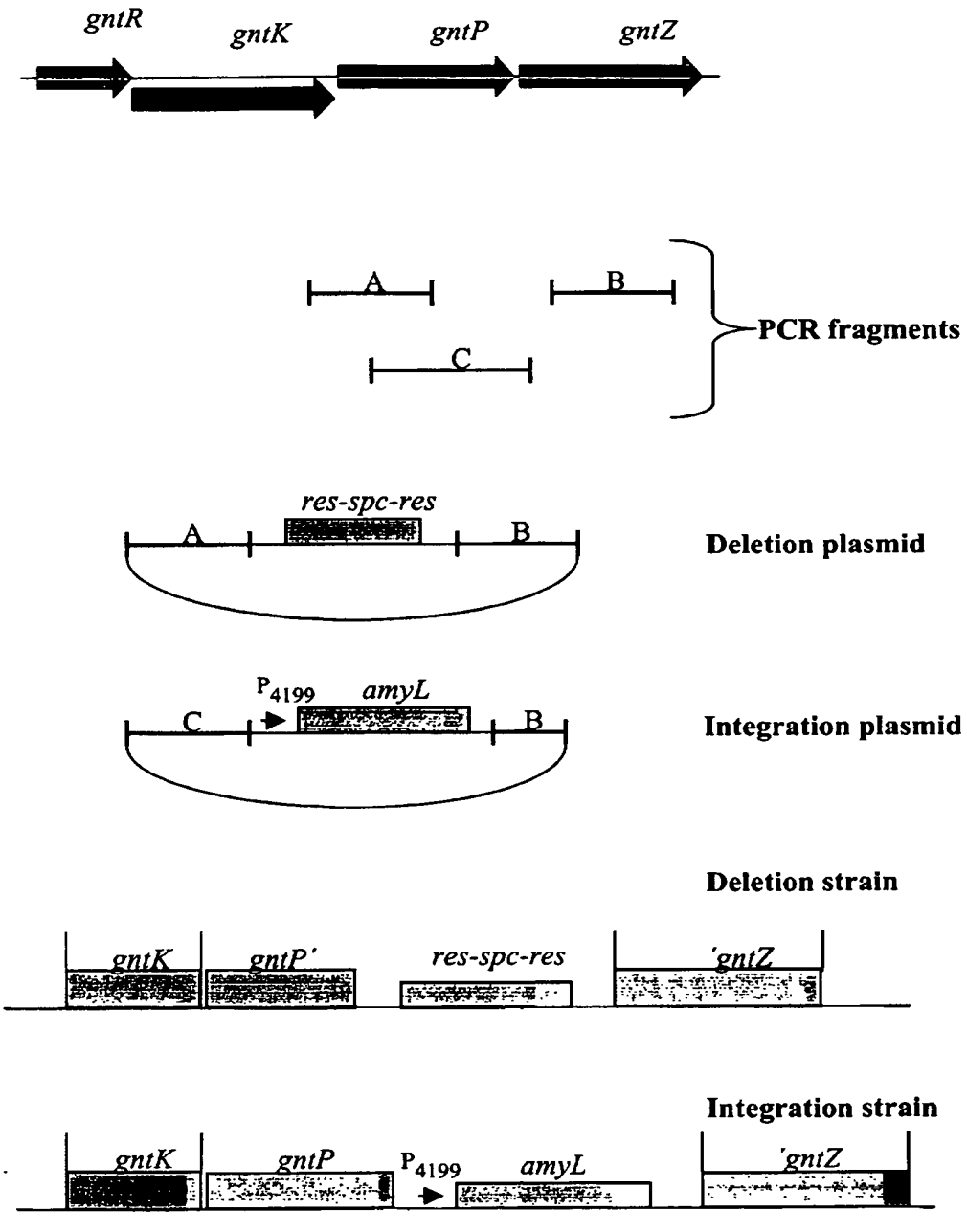
xylB

Fig 1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B
Integration plasmid
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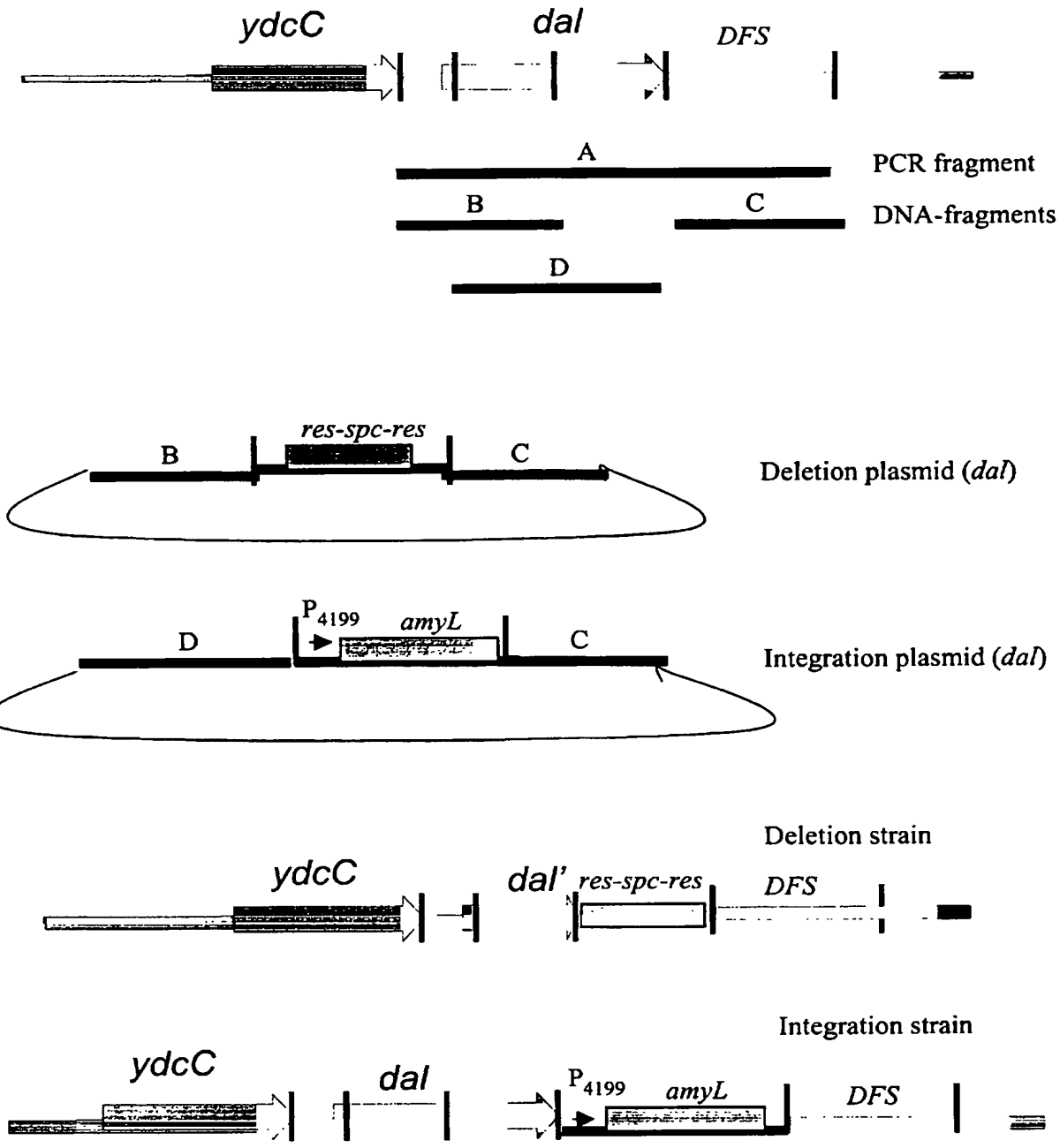
Fig 2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DFS
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amyL
D
C
Integration plasmid (dal)
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dal
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amyL
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Fig 3

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ctt gtc ctc ggt gca gtg ccg acc gaa tat gca agc att gcc gcg gaa    1602
Leu Val Leu Gly Ala Val Pro Thr Glu Tyr Ala Ser Ile Ala Ala Glu
 85                  90                  95                  100

aag cgc att atc gtg act ggc tac tcc gtc ggc tgg ctg aaa gac gtg    1650
Lys Arg Ile Ile Val Thr Gly Tyr Ser Val Gly Trp Leu Lys Asp Val
                105                 110                 115

ctc ggt ttt ctg aat gag gcc gaa gct cct ctt gaa tat cat ttg aag    1698
Leu Gly Phe Leu Asn Glu Ala Glu Ala Pro Leu Glu Tyr His L u Lys
```

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                120                 125                 130

atc gac acg ggc atg ggc cgc ctt ggc tgc aaa acg gaa gaa gag atc   1746
Ile Asp Thr Gly Met Gly Arg Leu Gly Cys Lys Thr Glu Glu Glu Il
        135                 140                 145

aaa gaa atg atg gag atg acc gaa tcg aac gat aag ctc aat tgt acg   1794
Lys Glu Met Met Glu Met Thr Glu Ser Asn Asp Lys Leu Asn Cys Thr
    150                 155                 160

ggc gtg ttc act cat ttc gcc acg gcg gac gaa aag gac acc gat tat   1842
Gly Val Phe Thr His Phe Ala Thr Ala Asp Glu Lys Asp Thr Asp Tyr
165                 170                 175                 180

ttc aac atg cat ctt gac cgc ttt aaa gag ctg atc agc ccc ttc ccg   1890
Phe Asn Met His Leu Asp Arg Phe Lys Glu Leu Ile Ser Pro Phe Pro
                185                 190                 195

ctt gac cgt ttg atg gtg cat tcg tca aac agc gcc gcg ggt ctg cgc   1938
Leu Asp Arg Leu Met Val His Ser Ser Asn Ser Ala Ala Gly Leu Arg
            200                 205                 210

ttc agg gaa cag cta ttt aat gcc gtc cgc ttc ggc atc ggc atg tac   1986
Phe Arg Glu Gln Leu Phe Asn Ala Val Arg Phe Gly Ile Gly Met Tyr
        215                 220                 225

ggt ttg gcg ccg tca acc gaa ata aaa gac gag ctg ccg ttt cgt ctg   2034
Gly Leu Ala Pro Ser Thr Glu Ile Lys Asp Glu Leu Pro Phe Arg Leu
    230                 235                 240

cgg gaa gtg ttt tcg ctt cat acc gaa ctc acc cat gtg aaa aaa att   2082
Arg Glu Val Phe Ser Leu His Thr Glu Leu Thr His Val Lys Lys Ile
245                 250                 255                 260

aaa aaa ggc gag agc gtc agc tac ggg gcg aca tat aca gct cag cgc   2130
Lys Lys Gly Glu Ser Val Ser Tyr Gly Ala Thr Tyr Thr Ala Gln Arg
                265                 270                 275

gac gaa tgg atc ggg aca gtc ccc gtc ggg tat gcc gac gga tgg ctg   2178
Asp Glu Trp Ile Gly Thr Val Pro Val Gly Tyr Ala Asp Gly Trp Leu
            280                 285                 290

agg cgc ctg gcc gga acg gaa gtg ctg atc gac gga aaa cgc caa aaa   2226
Arg Arg Leu Ala Gly Thr Glu Val Leu Ile Asp Gly Lys Arg Gln Lys
        295                 300                 305

ata gca ggg aga atc tgc atg gac cag ttc atg att tcc ctt gcc gaa   2274
Ile Ala Gly Arg Ile Cys Met Asp Gln Phe Met Ile Ser Leu Ala Glu
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    310                 315                 320

gaa tac cct gtc ggc aca aag gtt acc ttg atc gga aag caa aaa gac   2322
Glu Tyr Pro Val Gly Thr Lys Val Thr Leu Il  Gly Lys Gln Lys Asp
325                 330                 335                 340

gaa tgg atc tca gtc gac gaa atc gcc caa aat ttg cag acg atc aat   2370
Glu Trp Ile Ser Val Asp Glu Ile Ala Gln Asn Leu Gln Thr Ile Asn
                345                 350                 355

tat gaa att acc tgt atg ata agt tca agg gtg ccc cgt atg ttt ttg   2418
Tyr Glu Ile Thr Cys Met Ile Ser Ser Arg Val Pro Arg Met Phe Leu
            360                 365                 370

gaa aat ggg agt ata atg gaa ata agg aat ccg atc ttg cct gat caa   2466
Glu Asn Gly Ser Ile Met Glu Ile Arg Asn Pro Ile Leu Pro Asp Gln
        375                 380                 385

tcc tgaaaattga tgaattagcg gaaaaacaac tttgcttgcg aaaagaataa        2519
Ser

tgatatgatt atgaatggaa tggatagagt gttgtatccg taagtttggt ggaggtgtat 2579

gtttttgtct gaatccagcg caacaactga aatattgatt cgcttgccag aagctttagt 2639

atcagaactg gatggtgtcg tcatgcgaga taaccgggag cagganatga actgatttta 2699

ccaagccaca aaaatgtagg aacgcgaacg caaaaaatcg acaaattcgg ggaatcgatg 2759

agaagcggtt atatggagat ggccaagatc caatttgaac atctcttctg aggctcaatt 2819

tgcagagtat gaggctgaaa acacagtaga gcgcttacta agcggatgat aatcatttga 2879

ttgttaaacg cggcgatgtt tattttgctg acctatctcc tgttgttggc tcagaacaag 2939

gcggggtgcg cccggtttta gtgattcaaa acaacatcgg caatcgcttc agcccaactg 2999

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cgattgacaa gcaaagatta acggacaaaa tcacccatct cgatgatgaa atgatggaaa 3179

aggtcaacga agccttacaa atcagtttgg cactcattga tttttaatat tgatgaaagt 3239

tgctcgaggc gaaagagcaa ctttttttgt gttcaaaaat aacaatacga tataatggta 3299

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<210> 13
<211> 389
<212> PRT
<213> Bacillus licheniformis

<400> 13

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Met Ser Leu Lys Pro Phe Tyr Arg Lys Thr Trp Ala Glu Ile Asp Leu
  1               5                  10                  15

Thr Ala Leu Lys Glu Asn Val Arg Asn Met Lys Arg His Ile Gly Glu
             20                  25                  30

His Val Arg Leu Met Ala Val Val Lys Ala Asn Ala Tyr Gly His Gly
         35                  40                  45

Asp Ala Gln Val Ala Lys Ala Ala Leu Ala Glu Gly Ala Ser Ile Leu
     50                  55                  60

Ala Val Ala Leu Leu Asp Glu Ala Leu Ser Leu Arg Ala Gln Gly Ile
 65                  70                  75                  80

Glu Glu Pro Ile Leu Val Leu Gly Ala Val Pro Thr Glu Tyr Ala Ser
                 85                  90                  95

Ile Ala Ala Glu Lys Arg Ile Ile Val Thr Gly Tyr Ser Val Gly Trp
            100                 105                 110

Leu Lys Asp Val Leu Gly Phe Leu Asn Glu Ala Glu Ala Pro Leu Glu
        115                 120                 125

Tyr His Leu Lys Ile Asp Thr Gly Met Gly Arg Leu Gly Cys Lys Thr
    130                 135                 140

Glu Glu Glu Ile Lys Glu Met Met Glu Met Thr Glu Ser Asn Asp Lys
145                 150                 155                 160

Leu Asn Cys Thr Gly Val Phe Thr His Phe Ala Thr Ala Asp Glu Lys
                165                 170                 175

Asp Thr Asp Tyr Phe Asn Met His Leu Asp Arg Phe Lys Glu Leu Ile
            180                 185                 190

Ser Pro Phe Pro Leu Asp Arg Leu Met Val His Ser Ser Asn Ser Ala
        195                 200                 205

Ala Gly Leu Arg Phe Arg Glu Gln Leu Phe Asn Ala Val Arg Phe Gly
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210 215 220

Ile Gly Met Tyr Gly Leu Ala Pro Ser Thr Glu Ile Lys Asp Glu Leu
225 230 235 240

Pro Phe Arg Leu Arg Glu Val Phe Ser Leu His Thr Glu Leu Thr His
245 250 255

Val Lys Lys Ile Lys Lys Gly Glu Ser Val Ser Tyr Gly Ala Thr Tyr
260 265 270

Thr Ala Gln Arg Asp Glu Trp Ile Gly Thr Val Pro Val Gly Tyr Ala
275 280 285

Asp Gly Trp Leu Arg Arg Leu Ala Gly Thr Glu Val Leu Ile Asp Gly
290 295 300

Lys Arg Gln Lys Ile Ala Gly Arg Ile Cys Met Asp Gln Phe Met Ile
305 310 315 320

Ser Leu Ala Glu Glu Tyr Pro Val Gly Thr Lys Val Thr Leu Ile Gly
325 330 335

Lys Gln Lys Asp Glu Trp Ile Ser Val Asp Glu Ile Ala Gln Asn Leu
340 345 350

Gln Thr Ile Asn Tyr Glu Ile Thr Cys Met Ile Ser Ser Arg Val Pro
355 360 365

Arg Met Phe Leu Glu Asn Gly Ser Ile Met Glu Ile Arg Asn Pro Ile
370 375 380

Leu Pro Asp Gln Ser
385

<210> 14
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer # 148779; [Upstream of a SphI site in the dal regi n]

<400> 14

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<210> 15
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer #
148780: [BamHI < dal]

<400> 15
aaaggatccc cctgactaca tctggc 26

<210> 16
<211> 39
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer #
170046 [NotI; < ermC gene and the replication
origin of pE194>]

<400> 16
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<210> 17
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer #
170047 [EcoRI; <- ermC gene and the replication
origin of pE194->]

<400> 17
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<210> 18
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
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<400> 18
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<210> 19
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<212> DNA
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<220>
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150507 [KpnI; <- dal gene]

<400> 19
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<210> 20
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<223> Description of Artificial Sequence: Primer #
158089 [KpnI; < T1 and T2 Terminators of rrnB]

<400> 20
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<210> 21
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<212> DNA
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<400> 21
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<210> 22
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
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150508 [HindIII; < DFS]

<400> 22
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<210> 23
<211> 30
<212> DNA
<213> Artificial Sequence

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150509 [NheI; < DFS]

<400> 23
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<210> 24
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<212> DNA
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<220>
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145507 [< ydcC - dal - DFS >]

<400> 24
gcgtaccgtt aaagtcgaac agcg 24

<210> 25
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<212> DNA
<213> Artificial Sequence

<220>
<223> Descripti n f Artificial Sequence: Primer #
150509 [NheI; < ydcC - dal - DFS >]

<400> 25
aaagctagca tccccctgac tacatctggc 30